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<p>(54) Title: VASCULAR ENDOTHELIAL CELL GROWTH FACTOR ANTAGONISTS AND USES THEREOF</p> <p>(57) Abstract</p> <p>The present invention provides vascular endothelial cell growth factor (VEGF) antagonists and methods of using VEGF antagonists. VEGF antagonists contemplated by the invention include VEGF antibodies and VEGF receptor fusion proteins. Methods of treating edema and stroke using VEGF antagonists are also provided.</p> <p style="text-align: right;">BEST AVAILABLE COPY</p>		

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VASCULAR ENDOTHELIAL CELL GROWTH FACTOR ANTAGONISTS AND USES THEREOF

Field of the Invention

The present invention relates to vascular endothelial cell growth factor (VEGF) antagonists, to therapeutic compositions comprising the antagonists, and to methods of use of the antagonists for diagnostic and therapeutic purposes. In particular, the present invention relates to methods of treatment of stroke or edema using VEGF antagonists.

Background of the Invention

The two major cellular components of the vasculature are the endothelial and smooth muscle cells. The endothelial cells form the lining of the inner surface of all blood vessels, and constitute a nonthrombogenic interface between blood and tissue. In addition, endothelial cells are an important component for the development of new capillaries and blood vessels. Thus, endothelial cells proliferate during the angiogenesis, or neovascularization, associated with tumor growth and metastasis, as well as a variety of non-neoplastic diseases or disorders.

Various naturally occurring polypeptides reportedly induce the proliferation of endothelial cells. Among those polypeptides are the basic and acidic fibroblast growth factors (FGF), Burgess and Maciag, Annual Rev. Biochem., 58:575 (1989), platelet-derived endothelial cell growth factor (PD-ECGF), Ishikawa, *et al.*, Nature, 338:557 (1989), and vascular endothelial growth factor (VEGF), Leung, *et al.*, Science 246:1306 (1989); Ferrara & Henzel, Biochem. Biophys. Res. Commun. 161:851 (1989); Tischer, *et al.*, Biochem. Biophys. Res. Commun. 165:1198 (1989); Ferrara, *et al.*, PCT Pat. Pub. No. WO 90/13649 (published November 15, 1990).

VEGF was first identified in media conditioned by bovine pituitary follicular or folliculostellate cells. Biochemical analyses indicate that bovine VEGF is a dimeric protein with an apparent molecular mass of approximately 45,000 Daltons, and with an apparent mitogenic specificity for vascular endothelial cells. DNA encoding bovine VEGF was isolated by screening a cDNA library prepared from such cells, using oligonucleotides based on the amino-terminal amino acid sequence of the protein as hybridization probes.

Human VEGF was obtained by first screening a cDNA library prepared from human cells, using bovine VEGF cDNA as a hybridization probe. One cDNA identified thereby encodes a 165-amino acid protein having greater than 95% homology to bovine VEGF, which protein is referred to as human VEGF (hVEGF). The mitogenic activity of human VEGF was confirmed by expressing the human VEGF cDNA in mammalian host cells. Media conditioned by cells transfected with the human VEGF cDNA promoted the proliferation of capillary endothelial cells, whereas control cells did not. See, Leung, *et al.*, Science 246:1306 (1989).

Several additional cDNAs were identified in human cDNA libraries that encode 121-, 189-, and 206-amino acid isoforms of hVEGF (also collectively referred to as hVEGF-related proteins). The 121-amino acid protein differs from hVEGF by virtue of the deletion of the 44 amino acids between residues 116 and 159 in hVEGF. The 189-amino acid protein differs from hVEGF by virtue of the insertion of 24 amino acids at residue 116 in hVEGF, and apparently is identical to human vascular permeability factor (hVFPF). The 206-amino acid protein differs from hVEGF by virtue of an insertion of 41 amino acids at residue 116 in hVEGF. Houck, *et al.*, Mol. Endocrin. 5:1806 (1991); Ferrara, *et al.*, J. Cell. Biochem. 47:211 (1991); Ferrara, *et al.*, Endocrine Reviews 13:18 (1992); Keck, *et al.*, Science 246:1309 (1989); Connolly, *et al.*, J. Biol. Chem. 264:20017 (1989); Keck, *et al.*, EPO Pat. Pub. No. 0 370 989 (published May 30, 1990).

Receptors for VEGF have been described in the literature. Two such receptors, flt-1 and flk-1, have been found to mediate VEGF effects [DeVries *et al.*, Science 255:989 (1992); Shibuya *et al.*, Oncogene 5:519 (1990); Matthews *et al.*, Proc. Natl. Acad. Sci. 88:9026 (1991); Terman *et al.*, Oncogene 6:1677 (1991); Terman *et al.*, Biochem. Biophys. Res. Comm. 187:1579 (1992); Neufeld *et al.*, Prog. Growth Factor Res. 5:89-97 (1994); Waltenberger *et al.*, J. Biol. Chem. 269:26988 (1994); Quinn *et al.*, Proc. Natl. Acad. Sci. 90:7533 (1993)], but their regulation and mechanisms are not yet fully understood. Lennmyr *et al.*, J. Neuropathology and Exp. Neurology 57:874-882 (1998). Both the flt-1 and flk-1 receptors are membrane-spanning receptors and belong to the class III tyrosine kinase receptor family. Barleon *et al.*, J. Cell Biochem. 54:56 (1994); Neufeld *et al.*, *supra*.

VEGF not only stimulates vascular endothelial cell proliferation, but also induces angiogenesis. Angiogenesis, which involves the

formation of new blood vessels from preexisting endothelium, is an important component of a variety of diseases and disorders including tumor growth and metastasis, rheumatoid arthritis, psoriasis, atherosclerosis, diabetic retinopathy, retrolental fibroplasia, neovascular glaucoma, age-related macular degeneration, hemangiomas, immune rejection of transplanted corneal tissue and other tissues, and chronic inflammation.

In the case of tumor growth, angiogenesis appears to be crucial for the transition from hyperplasia to neoplasia, and for providing nourishment to the growing solid tumor. Folkman, *et al.*, *Nature* 339:58 (1989). Angiogenesis also allows tumors to be in contact with the vascular bed of the host which may provide a route for metastasis of the tumor cells. Evidence for the role of angiogenesis in tumor metastasis is provided, for example, by studies showing a correlation between the number and density of microvessels in histologic sections of invasive human breast carcinoma and actual presence of distant metastases. Weidner, *et al.*, *New Engl. J. Med.* 324:1 (1991).

VEGF has also been reported to be involved in endothelial and vascular permeability. See, Ferrara *et al.*, *Endocrine Reviews* 18:4-25 (1997); Dobrogowska *et al.*, *J. Neurocytology* 27:163 (1998). Although not fully understood, VEGF is believed to increase endothelial cell leakage in skin, retina, and tumor tissues. Collins *et al.*, *Brit. J. Pharmacology* 109:195 (1993); Connolly *et al.*, *J. Clin. Invest.* 84:1470 (1989); Shweiki *et al.*, *Nature* 359:843 (1992); Monacci *et al.*, *Am. J. Physiol.* 264:C995 (1993); Stone *et al.*, *J. Neurosci.* 15:4738 (1995); Detmar *et al.*, *J. Invest. Dermatol.* 108:263 (1997); Weindel *et al.*, *Neurosurgery* 35:437 (1994). The potential effects and role of VEGF (and its receptors, particularly, the *flt-1* receptor), on endothelial cell and blood-brain barrier permeability have also been examined. See, e.g., Rosenstein *et al.*, *Proc. Natl. Acad. Sci.* 95:7086 (1998); Dobrogowska, *supra*; Kovacs *et al.*, *Stroke* 27:1865 (1996). Relatively diffuse VEGF mRNA expression has been observed in adult rat brain but at somewhat low abundance. Monacci *et al.*, *Am. J. Physiol.* 146:368-378 (1993). However, reduced oxygen tension has been shown to trigger VEGF expression [Dor and Keshet, *Trends in Cardiovascular Med.*, 7:289-294 (1998)] and enhanced levels of VEGF, *flt-1*, and *flk-1* have been shown to occur in the rat brain following the induction of focal cerebral ischemia. Hayashi *et al.*, *Stroke* 28:2039 (1997); Kovacs *et al.*, *supra*; Lennmyr *et al.*, *J.*

Neuropathology and Experimental Neurology, 57:874 (1998). The role of VEGF in the pathogenesis of stroke and BBB breakdown has been unclear with contradictory experimental observations cited in the literature.

For example, Nag et al., J. Neuropathology and Experimental Neurology 56:912 (1997), in their cortical cold-injury rat model, demonstrated the presence of mural VEGF in permeable pial vessels and arterioles within the damaged tissue and, from this observation, it was inferred that VEGF is one of several factors that may mediate BBB breakdown and edema formation. On the other hand, in Hayashi et al., J. Cerebral Blood Flow and Metabolism, 18:887 (1998), it is reported that VEGF itself, when applied topically on the surface of a reperfused rat brain after transient cerebral artery occlusion, reduced ischemic brain damage, infarct volume and edema formation.

Summary of the Invention

The present invention provides antagonists of VEGF, including (a) antibodies and variants thereof which are capable of specifically binding to hVEGF, hVEGF receptor, or a complex comprising hVEGF in association with hVEGF receptor, (b) hVEGF receptor and variants thereof, and (c) hVEGF variants. The antagonists inhibit, sequester or neutralize the mitogenic, angiogenic, vascular permeability or other biological activity of hVEGF, and thus are useful for the treatment of diseases or conditions characterized by undesirable excessive neovascularization, including by way of example, tumors, and especially solid malignant tumors, rheumatoid arthritis, psoriasis, atherosclerosis, diabetic and other retinopathies, retrolental fibroplasia, age-related macular degeneration, neovascular glaucoma, hemangiomas, thyroid hyperplasias (including Grave's disease), corneal and other tissue transplantation, and chronic inflammation. The antagonists also are useful for the treatment of diseases or conditions such as edema which may be associated with, e.g., tumors, stroke, head trauma, ascites associated with malignancies, Meigs' syndrome, lung inflammation, nephrotic syndrome, pericardial effusion (such as that associated with pericarditis), and pleural effusion.

In other aspects, the VEGF antagonists are polyspecific monoclonal antibodies which are capable of binding to (a) a non-hVEGF epitope, for example, an epitope of a protein involved in thrombogenesis or thrombolysis, or a tumor cell surface antigen, or to (b) hVEGF, hVEGF

receptor, or a complex comprising hVEGF in association with hVEGF receptor.

In still other aspects, the VEGF antagonists are conjugated with a cytotoxic moiety.

5 In another aspect, the invention concerns isolated nucleic acids encoding the monoclonal antibodies as hereinbefore described, and hybridoma cell lines which produce such monoclonal antibodies.

In another aspect, the invention concerns compositions, such as pharmaceutical compositions, comprising a VEGF antagonist in an amount
10 effective in reducing or eliminating hVEGF-mediated mitogenic, angiogenic, or other biological activity in a mammal.

In a different aspect, the invention concerns methods of treatment comprising administering to a mammal, preferably a human patient in need of such treatment, an effective amount of a VEGF antagonist. If desired,
15 the VEGF antagonist is co-administered, either simultaneously or sequentially, with one or more other VEGF antagonists, anti-tumor or anti-angiogenic substances, or therapies suitable for the disease or condition being treated.

In another aspect, the invention concerns a method for detecting
20 hVEGF in a test sample by means of contacting the test sample with an antibody capable of binding specifically to hVEGF and determining the extent of such binding.

Brief Description of the Drawings

25 Figure 1 shows the effect of anti-hVEGF monoclonal antibodies (A4.6.1 or B2.6.2) or an irrelevant anti-hepatocyte growth factor antibody (anti-HGF) on the binding of the anti-hVEGF monoclonal antibodies to hVEGF.

Figure 2 shows the effect of anti-hVEGF monoclonal antibodies
30 (A4.6.1 or B2.6.2) or an irrelevant anti-HGF antibody on the biological activity of hVEGF in cultures of bovine adrenal cortex capillary endothelial (ACE) cells.

Figure 3 shows the effect of anti-hVEGF monoclonal antibodies (A4.6.1, B2.6.2, or A2.6.1) on the binding of hVEGF to bovine ACE cells.

35 Figure 4 shows the effect of A4.6.1 anti-hVEGF monoclonal antibody treatment on the rate of growth of growth of NEG55 tumors in mice.

Figure 5 shows the effect of A4.6.1 anti-hVEGF monoclonal antibody treatment on the size of NEG55 tumors in mice after five weeks of treatment.

Figure 6 shows the effect of A4.6.1 anti-hVEGF monoclonal antibody (VEGF Ab) treatment on the growth of SK-LMS-1 tumors in mice.

Figure 7 shows the effect of varying doses of A4.6.1 anti-hVEGF monoclonal antibody (VEGF Ab) treatment on the growth of A673 tumors in mice.

Figure 8 shows the effect of A4.6.1 anti-hVEGF monoclonal antibody on the growth and survival of NEG55 (G55) glioblastoma cells in culture.

Figure 9 shows the effect of A4.6.1 anti-hVEGF monoclonal antibody on the growth and survival of A673 rhabdomyosarcoma cells in culture.

Figure 10 shows the effect of A4.6.1 anti-hVEGF monoclonal antibody on human synovial fluid-induced chemotaxis of human endothelial cells.

Figure 11 shows the effect of flt-IgG treatment on the extent of edematous tissue as depicted by high signal intensity on the T2-weighted MR image.

Figure 12 shows representative T2-weighted MR images recorded 24 hours following onset of ischemia for both the control (top panel) and treatment group (bottom panel), showing reduction in edematous tissue in the treatment group.

Figure 13 shows the effect of flt-IgG treatment on the size of infarction determined using high resolution anatomical MRI 8-12 weeks following onset of ischemia.

Figures 14A-B show an alignment of the amino acid sequences for the light and heavy variable domains respectively of affinity matured anti-VEGF antibodies compared to the F(ab)-12 antibody (SEQ ID NO:1). CDRs are underlined and designated by L, light, or H, heavy chains, and numbers 1-3. The affinity matured sequences are designated YO243-1 (SEQ ID NO:2); YO238-3 (SEQ ID NO:3); YO313-1 (SEQ ID NO:4); and YO317 (SEQ ID NO:5). Differences from F(ab)-12 are shown in shaded boxes.

Figures 15A-B show an alignment of the amino acid sequences for the light and heavy variable domains respectively of affinity matured anti-VEGF antibodies compared to the F(ab)-12 antibody (SEQ ID NO:1). CDRs are underlined and designated by L, light, or H, heavy chains, and numbers 1-3. The affinity matured sequences are designated YO192 (SEQ ID NO:6); YO238-3 (SEQ ID NO:3); YO239-19 (SEQ ID NO:7); and YO313-2 (SEQ ID NO:8). Differences from F(ab)-12 are shown in shaded boxes.

Detailed Description of the Invention

The present invention provides antagonists of hVEGF which are capable of inhibiting, sequestering, or neutralizing one or more of the biological activities of hVEGF. Antagonists of hVEGF act by interfering with the binding of hVEGF to a cellular receptor, by incapacitating or killing cells which have been activated by hVEGF, or by interfering with vascular endothelial cell activation after hVEGF binding to a cellular receptor. All such points of intervention by an hVEGF antagonist shall be considered equivalent for purposes of this invention. Thus, included within the scope of the invention are antibodies, monoclonal antibodies and humanized antibodies, or fragments thereof, that bind to hVEGF, hVEGF receptor, or a complex comprising hVEGF in association with hVEGF receptor. Also included within the scope of the invention are fragments and amino acid sequence variants of hVEGF that bind to hVEGF receptor but which do not exhibit a biological activity of native hVEGF. Also included within the scope of the invention are hVEGF receptor and fragments and amino acid sequence variants thereof which are capable of binding hVEGF.

The term "hVEGF" as used herein refers to the 165-amino acid human vascular endothelial cell growth factor, and related 121-, 189-, and 206-amino acid vascular endothelial cell growth factors, as described by Leung, *et al.*, Science 246:1306 (1989), and Houck, *et al.*, Mol. Endocrin. 5:1806 (1991), together with the naturally occurring allelic and processed forms of those growth factors.

The term "hVEGF receptor" or "hVEGFr" as used herein refers to a cellular receptor for hVEGF, ordinarily a cell-surface receptor found on vascular endothelial cells, as well as fragments and variants thereof which retain the ability to bind hVEGF. Typically, the hVEGF receptors and fragments and variants thereof that are hVEGF antagonists will be in isolated form, rather than being integrated into a cell membrane or fixed to a cell surface as may be the case in nature. One example of a hVEGF receptor is the *fms*-like tyrosine kinase (*flt* or *flt-1*), a transmembrane receptor in the tyrosine kinase family. DeVries, *et al.*, Science 255:989 (1992); Shibuya, *et al.*, Oncogene 5:519 (1990). The full length *flt* receptor comprises an extracellular domain, a transmembrane domain, and an intracellular domain with tyrosine kinase activity. The extracellular domain is involved in the binding of hVEGF, whereas the intracellular domain is involved in signal transduction.

Another example of a hVEGF receptor is the flk-1 receptor (also referred to as KDR). Matthews, et al., Proc. Nat. Acad. Sci. 88:9026 (1991); Terman, et al., Oncogene 6:1677 (1991); Terman, et al., Biochem. Biophys. Res. Commun. 187:1579 (1992).

5 Binding of hVEGF to the flt receptor results in the formation of at least two high molecular weight complexes, having apparent molecular weight of 205,000 and 300,000 Daltons. The 300,000 Dalton complex is believed to be a dimer comprising two receptor molecules bound to a single molecule of hVEGF.

10 Variants of hVEGFr also are included within the scope hereof. Representative examples include truncated forms of a receptor in which at least the transmembrane and cytoplasmic domains are deleted from the full length receptor molecule, and fusions proteins in which non-hVEGFr polymers or polypeptides are conjugated to the hVEGFr or, preferably, 15 truncated forms thereof. An example of such a non-hVEGF polypeptide is an immunoglobulin. In that case, for example, an extracellular domain sequence of the hVEGFr is substituted for the Fv domain of an immunoglobulin light or (preferably) heavy chain, with the C-terminus of the receptor extracellular domain covalently joined to the amino terminus 20 of the CH1, hinge, CH2 or other fragment of the heavy chain. Such variants are made in the same fashion as known immunoadhesins. See e.g., Gascoigne, et al., Proc. Nat. Acad. Sci. 84:2936 (1987); Capon, et al., Nature 337:525 (1989); Aruffo, et al., Cell 61:1303 (1990); Ashkenazi, et al., Proc. Nat. Acad. Sci. 88:10535 (1991); Bennett, et al., J. Biol. 25 Chem. 266:23060 (1991). Examples of various flt-IgG fusion proteins are described in Example 3 below. Truncated forms of the extracellular domain of the hVEGF receptor contemplated for use in the invention include ECD fragments (for instance, having one or more amino acids in the ECD sequence deleted) and ECD forms having one or more 30 immunoglobulin-like domains in the ECD deleted. Example 3B describes, for instance, a truncated ECD form which includes only the first three immunoglobulin-like domains of flt fused to a Fc-IgG. Preferably, a truncated form of the ECD used in making an antagonist molecule will include sufficient immunoglobulin-like domain(s) to ensure a desired 35 binding to hVEGF.

In other embodiments, the hVEGFr or fragments or variants thereof are conjugated to a non-proteinaceous polymer such as polyethylene glycol (PEG) (see e.g., Davis, et al., U.S. Patent No. 4,179,337; Goodson, et

al., BioTechnology 8:343-346 (1990); Abuchowski, et al., J. Biol. Chem. 252:3578 (1977); Abuchowski, et al., J. Biol. Chem. 252:3582 (1977) or carbohydrates (see e.g., Marshall, et al., Arch. Biochem. Biophys., 167:77 (1975)). This can serve to extend the biological half-life of the hVEGFr and reduce the possibility that the receptor will be immunogenic in the mammal to which it is administered.

The hVEGFr is used in substantially the same fashion as antibodies to hVEGF, taking into account the affinity of the antagonist and its valency for hVEGF. An extracellular domain sequence of hVEGF receptor, either by itself or fused to an immunoglobulin polypeptide or other carrier polypeptide, is especially useful as an antagonist of hVEGF, by virtue of its ability to sequester hVEGF that is present in a host but that is not bound to hVEGFr on a cell surface.

HVEGFr and fragments and variants thereof also are useful in screening assays to identify agonists and antagonists of hVEGF. For example, host cells transfected with DNA encoding hVEGFr (for example, flt or flk-1) overexpress the receptor polypeptide on the cell surface, making such recombinant host cells ideally suited for analyzing the ability of a test compound (for example, a small molecule, linear or cyclic peptide, or polypeptide) to bind to hVEGFr. hVEGFr and hVEGFr fusion proteins, such as an hVEGFr-IgG fusion protein, may be used in a similar fashion. For example, the fusion protein is bound to an immobilized support and the ability of a test compound to displace radiolabeled hVEGF from the hVEGFr domain of the fusion protein is determined.

The term "recombinant" used in reference to hVEGF, hVEGF receptor, antibodies, or other proteins, refers to proteins that are produced by recombinant DNA expression in a host cell. The host cell may be prokaryotic (for example, a bacterial cell such as E. coli) or eukaryotic (for example, a yeast or a mammalian cell).

Antagonist Antibodies

The term "monoclonal antibody" as used herein refers to an antibody obtained from a population of substantially homogeneous antibodies, i.e., the individual antibodies comprising the population are identical in specificity and affinity except for possible naturally occurring mutations that may be present in minor amounts. It should be appreciated that as a result of such naturally occurring mutations and the like, a

monoclonal antibody composition of the invention, which will predominantly contain antibodies capable of specifically binding hVEGF, hVEGF_r, or a complex comprising hVEGF in association with hVEGF_r ("hVEGF-hVEGF_r complex"), may also contain minor amounts of other antibodies.

Thus, the modifier "monoclonal" indicates the character of the antibody as being obtained from such a substantially homogeneous population of antibodies, and is not to be construed as requiring production of the antibody by any particular method. For example, monoclonal antibodies of the invention may be made using the hybridoma method first described by Kohler & Milstein, Nature 256:495 (1975), or may be made by recombinant DNA methods. See, e.g., Cabilly, et al., U.S. Pat. No. 4,816,567.

In the hybridoma method, a mouse or other appropriate host animal is immunized with antigen by subcutaneous, intraperitoneal, or intramuscular routes to elicit lymphocytes that produce or are capable of producing antibodies that will specifically bind to the protein(s) used for immunization. Alternatively, lymphocytes may be immunized in vitro.

Lymphocytes then are fused with myeloma cells using a suitable fusing agent, such as polyethylene glycol, to form a hybridoma cell. Goding, Monoclonal Antibodies: Principles and Practice, pp.59-103 (Academic Press, 1986).

The antigen may be hVEGF, hVEGF_r, or hVEGF-hVEGF_r complex. The antigen optionally is a fragment or portion or variant of any one of hVEGF or hVEGF_r having one or more amino acid residues that participate in the binding of hVEGF to one of its receptors. For example, immunization with an extracellular domain sequence of an hVEGF_r (such as, a truncated hVEGF_r polypeptide lacking at least transmembrane and intracellular domains) will be especially useful in producing antibodies that are antagonists of hVEGF, since it is region(s) within the extracellular domain that are involved in hVEGF binding.

Monoclonal antibodies capable of binding hVEGF-hVEGF_r complex are useful, particularly if they do not also bind to non-associated (non-complexed) hVEGF and hVEGF_r. Such antibodies thus only bind to cells undergoing immediate activation by hVEGF and accordingly are not sequestered by free hVEGF or hVEGF_r as is normally found in a mammal. Such antibodies typically bind an epitope that spans one or more points of contact between the receptor and hVEGF. Such antibodies have been produced for other ligand receptor complexes and may be produced here in

the same fashion. These antibodies need not, and may not, neutralize or inhibit a biological activity of non-associated hVEGF or hVEGFr, whether or not the antibodies are capable of binding to non-associated hVEGF or hVEGFr.

5 The hybridoma cells thus prepared are seeded and grown in a suitable culture medium that preferably contains one or more substances that inhibit the growth or survival of the unfused, parental myeloma cells. For example, if the parental myeloma cells lack the enzyme hypoxanthine guanine phosphoribosyl transferase (HGPRT or HPRT), the
10 culture medium for the hybridomas typically will include hypoxanthine, aminopterin, and thymidine (HAT medium), which substances prevent the growth of HGPRT-deficient cells.

 Preferred myeloma cells are those that fuse efficiently, support stable high level expression of antibody by the selected antibody-
15 producing cells, and are sensitive to a medium such as HAT medium. Among these, preferred myeloma cell lines are murine myeloma lines, such as those derived from MOPC-21 and MPC-11 mouse tumors available from the Salk Institute Cell Distribution Center, San Diego, California USA, SP-2 cells available from the American Type Culture Collection, Manassas,
20 Virginia USA, and P3X63Ag8U.1 cells described by Yelton, et al., Curr. Top. Microbiol. Immunol. 81:1 (1978). Human myeloma and mouse-human heteromyeloma cell lines also have been described for the production of human monoclonal antibodies. Kozbor, J. Immunol. 133:3001 (1984); Brodeur, et al., Monoclonal Antibody Production Techniques and
25 Applications, pp.51-63 (Marcel Dekker, Inc., New York, 1987).

 Culture medium in which hybridoma cells are growing is assayed for production of monoclonal antibodies directed against the antigen. Preferably, the binding specificity of monoclonal antibodies produced by hybridoma cells is determined by immunoprecipitation or by an in vitro
30 binding assay, such as radioimmunoassay (RIA) or enzyme-linked immunoabsorbent assay (ELISA). The monoclonal antibodies of the invention are those that preferentially immunoprecipitate hVEGF, hVEGFr, or hVEGF-hVEGFr complex, or that preferentially bind to at least one of those antigens in a binding assay, and that are capable of inhibiting a
35 biological activity of hVEGF.

 After hybridoma cells are identified that produce antagonist antibodies of the desired specificity, affinity, and activity, the clones may be subcloned by limiting dilution procedures and grown by standard

methods. Goding, Monoclonal Antibodies: Principles and Practice, pp.59-104 (Academic Press, 1986). Suitable culture media for this purpose include, for example, Dulbecco's Modified Eagle's Medium or RPMI-1640 medium. In addition, the hybridoma cells may be grown in vivo as ascites tumors in an animal.

The monoclonal antibodies secreted by the subclones are suitably separated from the culture medium, ascites fluid, or serum by conventional immunoglobulin purification procedures such as, for example, protein A-Sepharose, hydroxylapatite chromatography, gel electrophoresis, dialysis, or affinity chromatography.

DNA encoding monoclonal antibodies of the invention is readily isolated and sequenced using conventional procedures (e.g., by using oligonucleotide probes that are capable of binding specifically to genes encoding the heavy and light chains of murine antibodies). The hybridoma cells of the invention serve as a preferred source of such DNA. Once isolated, the DNA may be placed into expression vectors, which are then transfected into host cells such as simian COS cells, Chinese Hamster ovary (CHO) cells, or myeloma cells that do not otherwise produce immunoglobulin protein, to obtain the synthesis of monoclonal antibodies in the recombinant host cells.

The DNA optionally may be modified in order to change the character of the immunoglobulin produced by its expression. For example, humanized forms of murine antibodies are produced by substituting a complementarity determining region (CDR) of the murine antibody variable domain for the corresponding region of a human antibody. In some embodiments, selected framework region (FR) amino acid residues of the murine antibody also are substituted for the corresponding amino acid residues in the human antibody. Carter, et al., Proc. Nat. Acad. Sci. 89:4285 (1992); Carter, et al., BioTechnology 10:163 (1992). Chimeric forms of murine antibodies also are produced by substituting the coding sequence for selected human heavy and light constant chain domains in place of the homologous murine sequences. Cabilly, et al., U.S. Pat. No. 4,816,567; Morrison, et al., Proc. Nat. Acad. Sci. 81:6851 (1984).

Particular humanized antibodies contemplated for use in the present invention include the humanized and affinity matured anti-hVEGF antibodies described in published PCT applications WO 98/54331 (published October 15, 1998) and WO 98/54332 (published October 15, 1998). Such humanized or affinity matured anti-VEGF antibodies may be prepared or

made using the methods and techniques described in WO 98/54331 and WO 98/54332. Preferably, the anti-hVEGF antibody comprises the humanized F(ab), designated as F(ab)-12, or the affinity matured antibody, designated as YO317, in the above referenced PCT applications. Figures 14A-B and 15A-B illustrate the amino acid sequences (light and heavy chains) for these anti-VEGF antibodies, along with other affinity matured anti-VEGF antibodies, designated as YO192; YO238-3; YO239-19; YO313-2; YO243-1; and YO313-1. All such anti-VEGF antibodies are contemplated for use in the methods described herein. As disclosed in these published PCT applications, several of the humanized and affinity matured antibodies were demonstrated to reduce or inhibit VEGF activity in different types of *in vitro* assays, and thus act as VEGF antagonists.

The antibodies included within the scope of the invention thus include variant antibodies, such as chimeric (including "humanized") antibodies and hybrid antibodies comprising immunoglobulin chains capable of binding hVEGF, hVEGF_r, or hVEGF-hVEGF_r complex, and a non-hVEGF epitope.

The antibodies herein include all species of origin, and immunoglobulin classes (e.g., IgA, IgD, IgE, IgG, and IgM) and subclasses, as well as antibody fragments (e.g., Fab, F(ab')₂, and Fv), so long as they are capable of binding hVEGF, hVEGF_r, or hVEGF-hVEGF_r complex, and are capable of antagonizing a biological activity of hVEGF.

In a preferred embodiment of the invention, the antibody will have an affinity for the immunizing antigen of at least about 10⁹ liters/mole, as determined, for example, by the Scatchard analysis of Munson & Pollard, Anal. Biochem. 107:220 (1980). Also, the monoclonal antibody typically will inhibit the mitogenic or angiogenic activity of hVEGF at least about 50%, preferably greater than 80%, and most preferably greater than 90%, as determined, for example, by an *in vitro* cell survival or proliferation assay, such as described in Example 2 or as described in WO 98/54331 and WO 98/54332.

For some therapeutic and diagnostic applications, it is desirable that the monoclonal antibody be reactive with fewer than all of the different molecular forms of hVEGF. For example, it may be desirable to have a monoclonal antibody that is capable of specifically binding to the 165-amino acid sequence hVEGF but not to the 121- or 189-amino acid sequence hVEGF polypeptides. Such antibodies are readily identified by

comparative ELISA assays or comparative immunoprecipitation of the different hVEGF polypeptides.

Conjugates with Cytotoxic Moieties

5 In some embodiments it is desirable to provide a cytotoxic moiety conjugated to a hVEGF-specific monoclonal antibody or to hVEGFr. In these embodiments the cytotoxin serves to incapacitate or kill cells which are expressing or binding hVEGF or its receptor. The conjugate is targeted to the cell by the domain which is capable of binding to hVEGF, hVEGFr, or hVEGF-hVEGFr complex. Thus, monoclonal antibodies that are capable of binding hVEGF, hVEGFr, or hVEGF-hVEGFr complex are conjugated to cytotoxins. Similarly, hVEGFr is conjugated to a cytotoxin. While the monoclonal antibodies optimally are capable of neutralizing the activity of hVEGF alone (without the cytotoxin), it is not necessary in 10 this embodiment that the monoclonal antibody or receptor be capable of any more than binding to hVEGF, hVEGFr, or hVEGF-hVEGFr complex. 15

Typically, the cytotoxin is a protein cytotoxin, e.g. diphtheria, ricin or Pseudomonas toxin, although in the case of certain classes of immunoglobulins the Fc domain of the monoclonal antibody itself may serve to provide the cytotoxin (e.g., in the case of IgG2 antibodies, which are capable of fixing complement and participating in antibody-dependent cellular cytotoxicity (ADCC)). However, the cytotoxin does not need to be proteinaceous and may include chemotherapeutic agents heretofore employed, for example, for the treatment of tumors. 20

25 The cytotoxin typically is linked to a monoclonal antibody or fragment thereof by a backbone amide bond within (or in place of part or all of) the Fc domain of the antibody. Where the targeting function is supplied by hVEGFr, the cytotoxic moiety is substituted onto any domain of the receptor that does not participate in hVEGF binding; preferably, the moiety is substituted in place of or onto the transmembrane and or cytoplasmic domains of the receptor. The optimal site of substitution will be determined by routine experimentation and is well within the ordinary skill. 30

Conjugates which are protein fusions are easily made in recombinant cell culture by expressing a gene encoding the conjugate. Alternatively, the conjugates are made by covalently crosslinking the cytotoxic moiety to an amino acid residue side chain or C-terminal carboxyl of the antibody or the receptor, using methods known per se such as disulfide 35

exchange or linkage through a thioester bond using for example iminothiolate and methyl-4-mercaptobutyrimadate.

Conjugates with other Moieties

5 The monoclonal antibodies and hVEGFr that are antagonists of hVEGF also can also be conjugated to substances that may not be readily classified as cytotoxins in their own right, but which augment the activity of the compositions herein. For example, monoclonal antibodies or hVEGFr capable of binding to hVEGF, hVEGFr, or hVEGF-hVEGFr complex
10 are fused with heterologous polypeptides, such as viral sequences, with cellular receptors, with cytokines such as TNF, interferons, or interleukins, with polypeptides having procoagulant activity, and with other biologically or immunologically active polypeptides. Such fusions are readily made by recombinant methods. Typically such non-
15 immunoglobulin polypeptides are substituted for the constant domain(s) of an anti-hVEGF or anti-hVEGF-hVEGFr complex antibody, or for the transmembrane and/or intracellular domain of an hVEGFr. Alternatively, they are substituted for a variable domain of one antigen binding site of an anti-hVEGF antibody described herein.

20 In preferred embodiments, such non-immunoglobulin polypeptides are joined to or substituted for the constant domains of an antibody described herein. Bennett, et al., J. Biol. Chem. 266:23060-23067 (1991). Alternatively, they are substituted for the Fv of an antibody herein to create a chimeric polyvalent antibody comprising at least one
25 remaining antigen binding site having specificity for hVEGF, hVEGFr, or a hVEGF-hVEGFr complex, and a surrogate antigen binding site having a function or specificity distinct from that of the starting antibody.

Heterospecific Antibodies

30 Monoclonal antibodies capable of binding to hVEGF, hVEGFr, or hVEGF-hVEGFr complex need only contain a single binding site for the enumerated epitopes, typically a single heavy-light chain complex or fragment thereof. However, such antibodies optionally also bear antigen binding domains that are capable of binding an epitope not found within
35 any one of hVEGF, hVEGFr, or hVEGF-hVEGFr complex. For example, substituting the corresponding amino acid sequence or amino acid residues of a native anti-hVEGF, anti-hVEGFr, or anti-hVEGF-hVEGFr complex

antibody with the complementarity-determining and, if necessary, framework residues of an antibody having specificity for an antigen other than hVEGF, hVEGFr, or hVEGF-hVEGFr complex will create a polyspecific antibody comprising one antigen binding site having specificity for hVEGF, hVEGFr, or hVEGF-hVEGFr complex, and another antigen binding site having specificity for the non-hVEGF, hVEGFr, or hVEGF-hVEGFr complex antigen. These antibodies are at least bivalent, but may be polyvalent, depending upon the number of antigen binding sites possessed by the antibody class chosen. For example, antibodies of the IgM class will be polyvalent.

In preferred embodiments of the invention such antibodies are capable of binding an hVEGF or hVEGFr epitope and either (a) a polypeptide active in blood coagulation, such as protein C or tissue factor, (b) a cytotoxic protein such as tumor necrosis factor (TNF), or (c) a non-hVEGFr cell surface receptor, such as CD4, or HER-2 receptor (Maddon, *et al.*, Cell 42:93 (1985); Coussens, *et al.*, Science 230:1137 (1985)). Heterospecific, multivalent antibodies are conveniently made by cotransforming a host cell with DNA encoding the heavy and light chains of both antibodies and thereafter recovering, by immunoaffinity chromatography or the like, the proportion of expressed antibodies having the desired antigen binding properties. Alternatively, such antibodies are made by *in vitro* recombination of monospecific antibodies.

Monovalent Antibodies

Monovalent antibodies capable of binding to hVEGFr or hVEGF-hVEGFr complex are especially useful as antagonists of hVEGF. Without limiting the invention to any particular mechanism of biological activity, it is believed that activation of cellular hVEGF receptors proceeds by a mechanism wherein the binding of hVEGF to cellular hVEGF receptors induces aggregation of the receptors, and in turn activates intracellular receptor kinase activity. Because monovalent anti-hVEGF receptor antibodies cannot induce such aggregation, and therefore cannot activate hVEGF receptor by that mechanism, they are ideal antagonists of hVEGF.

It should be noted, however, that these antibodies should be directed against the hVEGF binding site of the receptor or should otherwise be capable of interfering with hVEGF binding to the receptor hVEGF, such as by sterically hindering hVEGF access to the receptor. As described elsewhere herein, however, anti-hVEGFr antibodies that are not

capable of interfering with hVEGF binding are useful when conjugated to non-immunoglobulin moieties, for example, cytotoxins.

Methods for preparing monovalent antibodies are well known in the art. For example, one method involves recombinant expression of immunoglobulin light chain and modified heavy chain. The heavy chain is truncated generally at any point in the Fc region so as to prevent heavy chain crosslinking. Alternatively, the relevant cysteine residues are substituted with another amino acid residue or are deleted so as to prevent crosslinking. In vitro methods are also suitable for preparing monovalent antibodies. For example, Fab fragments are prepared by enzymatic cleavage of intact antibody.

Diagnostic Uses

For diagnostic applications, the antibodies or hVEGFr of the invention typically will be labeled with a detectable moiety. The detectable moiety can be any one which is capable of producing, either directly or indirectly, a detectable signal. For example, the detectable moiety may be a radioisotope, such as ^3H , ^{14}C , ^{32}P , ^{35}S , or ^{125}I , a fluorescent or chemiluminescent compound, such as fluorescein isothiocyanate, rhodamine, or luciferin; radioactive isotopic labels, such as, e.g., ^{125}I , ^{32}P , ^{14}C , or ^3H , or an enzyme, such as alkaline phosphatase, beta-galactosidase or horseradish peroxidase.

Any method known in the art for separately conjugating the antibody or hVEGFr to the detectable moiety may be employed, including those methods described by Hunter, et al., Nature 144:945 (1962); David, et al., Biochemistry 13:1014 (1974); Pain, et al., J. Immunol. Meth. 40:219 (1981); and Nygren, J. Histochem. and Cytochem. 30:407 (1982). The antibodies and receptors of the present invention may be employed in any known assay method, such as competitive binding assays, direct and indirect sandwich assays, and immunoprecipitation assays. Zola, Monoclonal Antibodies: A Manual of Techniques, pp.147-158 (CRC Press, Inc., 1987).

Competitive binding assays rely on the ability of a labeled standard (which may be hVEGF or an immunologically reactive portion thereof) to compete with the test sample analyte (hVEGF) for binding with a limited amount of antibody. The amount of hVEGF in the test sample is inversely proportional to the amount of standard that becomes bound to

the antibodies or receptors. To facilitate determining the amount of standard that becomes bound, the antibodies or receptors generally are insolubilized before or after the competition, so that the standard and analyte that are bound to the antibodies or receptors may conveniently be separated from the standard and analyte which remain unbound.

Sandwich assays involve the use of two antibodies or receptors, each capable of binding to a different immunogenic portion, or epitope, of the protein to be detected. In a sandwich assay, the test sample analyte is bound by a first antibody or receptor which is immobilized on a solid support, and thereafter a second antibody binds to the analyte, thus forming an insoluble three part complex. David & Greene, U.S. Pat No. 4,376,110. The second antibody or receptor may itself be labeled with a detectable moiety (direct sandwich assays) or may be measured using an anti-immunoglobulin antibody that is labeled with a detectable moiety (indirect sandwich assay). For example, one type of sandwich assay is an ELISA assay, in which case the detectable moiety is an enzyme.

The antibodies or receptor herein also is useful for in vivo imaging, wherein an antibody or hVEGFr labeled with a detectable moiety is administered to a patient, preferably into the bloodstream, and the presence and location of the labeled antibody or receptor in the patient is assayed. This imaging technique is useful, for example, in the staging and treatment of neoplasms. The antibody or hVEGFr is labeled with any moiety that is detectable in a mammal, whether by nuclear magnetic resonance, radiology, or other detection means known in the art.

Antagonist Variants of hVEGF

In addition to the antibodies described herein, other useful antagonists of hVEGF include fragments and amino acid sequence variants of native hVEGF that bind to hVEGF receptor but that do not exhibit the biological activity of native hVEGF. For example, such antagonists include fragments and amino acid sequence variants that comprise a receptor binding domain of hVEGF, but that lack a domain conferring biological activity, or that otherwise are defective in activating cellular hVEGF receptors, such as in the case of a fragment or an amino acid sequence variant that is deficient in its ability to induce aggregation or activation of cellular hVEGF receptors. The term "receptor binding domain" refers to the amino acid sequences in hVEGF

that are involved in hVEGF receptor binding. The term "biological activity domain" or "domain conferring biological activity" refers to an amino acid sequence in hVEGF that confers a particular biological activity of the factor, such as mitogenic, angiogenic, or vascular permeability activity.

The observation that hVEGF appears to be capable of forming a complex with two or more hVEGFr molecules on the surface of a cell suggests that hVEGF has at least two discrete sites for binding to hVEGFr and that it binds to such cellular receptors in sequential fashion, first at one site and then at the other before activation occurs, in the fashion of growth hormone, prolactin and the like (see e.g., Cunningham, et al., Science 254:821 (1991); deVos, et al., Science 255:306 (1992); Fuh, et al., Science 256:1677 (1992)). Accordingly, antagonist variants of hVEGF are selected in which one receptor binding site of hVEGF (typically the site involved in the initial binding of hVEGF to hVEGFr) remains unmodified (or if modified is varied to enhance binding), while a second receptor binding site of hVEGF typically is modified by nonconservative amino acid residue substitution(s) or deletion(s) in order to render that binding site inoperative.

Receptor binding domains in hVEGF and hVEGF binding domains in hVEGFr are determined by methods known in the art, including X-ray studies, mutational analyses, and antibody binding studies. The mutational approaches include the techniques of random saturation mutagenesis coupled with selection of escape mutants, and insertional mutagenesis. Another strategy suitable for identifying receptor-binding domains in ligands is known as alanine (Ala)-scanning mutagenesis. Cunningham, et al., Science 244, 1081-1985 (1989). This method involves the identification of regions that contain charged amino acid side chains. The charged residues in each region identified (i.e. Arg, Asp, His, Lys, and Glu) are replaced (one region per mutant molecule) with Ala and the receptor binding of the obtained ligands is tested, to assess the importance of the particular region in receptor binding. A further powerful method for the localization of receptor binding domains is through the use of neutralizing anti-hVEGF antibodies. Kim, et al., Growth Factors 7:53 (1992). Usually a combination of these and similar methods is used for localizing the domains involved in receptor binding.

The term "amino acid sequence variant" used in reference to hVEGF refers to polypeptides having amino acid sequences that differ to some

extent from the amino acid sequences of the native forms of hVEGF. Ordinarily, antagonist amino acid sequence variants will possess at least about 70% homology with at least one receptor binding domain of a native hVEGF, and preferably, they will be at least about 80%, more preferably at least about 90% homologous with a receptor binding domain of a native hVEGF. The amino acid sequence variants possess substitutions, deletions, and/or insertions at certain positions within the amino acid sequence of native hVEGF, such that the variants retain the ability to bind to hVEGF receptor (and thus compete with native hVEGF for binding to hVEGF receptor) but fail to induce one or more of the biological effects of hVEGF, such as endothelial cell proliferation, angiogenesis, or vascular permeability.

"Homology" is defined as the percentage of residues in the amino acid sequence variant that are identical with the residues in the amino acid sequence of a receptor binding domain of a native hVEGF after aligning the sequences and introducing gaps, if necessary, to achieve the maximum percent homology and not considering any conservative substitutions as part of the percentage of amino acid homology. Methods and computer programs for the alignment are well known in the art. One such computer program is "Align 2", authored by Genentech, Inc., which was filed with user documentation in the United States Copyright Office, Washington, DC 20559, on December 10, 1991. Those skilled in the art can determine, using routine skill, appropriate parameters for measuring alignment, including any algorithms needed to achieve maximal alignment over the length of the sequences being compared.

Substitutional variants are those that have at least one amino acid residue in a native sequence removed and a different amino acid inserted in its place at the same position. The substitutions may be single, where only one amino acid in the molecule has been substituted, or they may be multiple, where two or more amino acids have been substituted in the same molecule.

Insertional variants are those with one or more amino acids inserted immediately adjacent to an amino acid at a particular position in a native sequence. Immediately adjacent to an amino acid means connected to either the α -carboxy or α -amino functional group of the amino acid.

Deletional variants are those with one or more amino acid residues in a native sequence removed. Ordinarily, deletional variants will have

one or two amino acid residues deleted in a particular region of the molecule.

Fragments and amino acid sequence variants of hVEGF are readily prepared by methods known in the art, such as by site directed
5 mutagenesis of the DNA encoding the native factor. The mutated DNA is inserted into an appropriate expression vector, and host cells are then transfected with the recombinant vector. The recombinant host cells and grown in suitable culture medium, and the desired fragment or amino acid sequence variant expressed in the host cells then is recovered from the
10 recombinant cell culture by chromatographic or other purification methods.

Alternatively, fragments and amino acid variants of hVEGF are prepared in vitro, for example by proteolysis of native hVEGF, or by synthesis using standard solid-phase peptide synthesis procedures as
15 described by Merrifield (J. Am. Chem. Soc. 85:2149 (1963)), although other equivalent chemical syntheses known in the art may be used. Solid-phase synthesis is initiated from the C-terminus of the peptide by coupling a protected α -amino acid to a suitable resin. The amino acids are coupled to the peptide chain using techniques well known in the art
20 for the formation of peptide bonds.

Therapeutic Uses

The terms "treating" "treatment", "therapy" and "therapeutic" as used herein refer to curative therapy, prophylactic therapy and
25 preventative therapy.

For therapeutic applications, the antagonists of the invention are administered to a mammal, preferably a human, in an acceptable dosage form, including those that may be administered to a human intravenously as a bolus or by continuous infusion over a period of time, by
30 intramuscular, intraperitoneal, intra-cerebrospinal, subcutaneous, intra-articular, intrasynovial, intradural, intrathecal, oral, topical, or inhalation routes. The antagonists also are suitably administered by intratumoral, peritumoral, intralesional, or perilesional routes, to exert local as well as systemic therapeutic effects. The intraperitoneal
35 route is expected to be particularly useful, for example, in the treatment of ovarian tumors. Intravenous infusion is expected to be particularly useful for instance, in the treatment of cerebral edema.

Such dosage forms encompass carriers that are inherently nontoxic and nontherapeutic. Examples of such carriers include ion exchangers, alumina, aluminum stearate, lecithin, serum proteins, such as human serum albumin, buffer substances such as phosphates, glycine, sorbic acid, potassium sorbate, partial glyceride mixtures of saturated vegetable fatty acids, water, salts, or electrolytes such as protamine sulfate, disodium hydrogen phosphate, potassium hydrogen phosphate, sodium chloride, zinc salts, colloidal silica, magnesium trisilicate, polyvinyl pyrrolidone, cellulose-based substances, and polyethylene glycol.

Carriers for topical or gel-based forms of antagonist include polysaccharides such as sodium carboxymethylcellulose or methylcellulose, polyvinylpyrrolidone, polyacrylates, polyoxyethylene-polyoxypropylene-block polymers, polyethylene glycol, and wood wax alcohols. Conventional depot forms can be suitably used. Such forms include, for example, microcapsules, nano-capsules, liposomes, plasters, inhalation forms, nose sprays, sublingual tablets, and sustained-release preparations. The antagonist will typically be formulated in such vehicles at a concentration of about 0.1 mg/ml to 100 mg/ml.

Suitable examples of sustained release preparations include semipermeable matrices of solid hydrophobic polymers containing the antagonist, which matrices are in the form of shaped articles, e.g. films, or microcapsules. Examples of sustained-release matrices include polyesters, hydrogels (for example, poly(2-hydroxyethyl-methacrylate) as described by Langer *et al.*, J. Biomed. Mater. Res. 15:167 (1981) and Langer, Chem. Tech., 12: 98-105 (1982), or poly(vinylalcohol), polylactides (U.S. Pat. No. 3,773,919), copolymers of L-glutamic acid and gamma ethyl-L-glutamate (Sidman *et al.*, Biopolymers, 22:547 (1983), non-degradable ethylene-vinyl acetate (Langer *et al.*, *supra*), degradable lactic acid-glycolic acid copolymers such as the Lupron Depot™ (injectable micropheres composed of lactic acid-glycolic acid copolymer and leuprolide acetate), and poly-D-(-)-3-hydroxybutyric acid. While polymers such as ethylene-vinyl acetate and lactic acid-glycolic acid enable release of molecules for over 100 days, certain hydrogels release proteins for shorter time periods. When encapsulated polypeptide antagonists remain in the body for a long time, they may denature or aggregate as a result of exposure to moisture at 37°C, resulting in a loss of biological activity and possible changes in immunogenicity. Rational strategies can be devised for stabilization depending on the

mechanism involved. For example, if the aggregation mechanism is discovered to be intermolecular S-S bond formation through thio-disulfide interchange, stabilization may be achieved by modifying sulfhydryl residues, lyophilizing from acidic solutions, controlling moisture content, using appropriate additives, and developing specific polymer matrix compositions.

Sustained-release hVEGF antagonist compositions also include liposomally entrapped antagonist antibodies or hVEGF_r. Liposomes containing the antagonists are prepared by methods known in the art, such as described in Epstein, et al., Proc. Natl. Acad. Sci. USA, 82:3688 (1985); Hwang, et al., Proc. Natl. Acad. Sci. USA, 77:4030 (1980); U.S. Patent No. 4,485,045; U.S. Patent No. 4,544,545. Ordinarily the liposomes are the small (about 200-800 Angstroms) unilamellar type in which the lipid content is greater than about 30 mol.% cholesterol, the selected proportion being adjusted for the optimal HRG therapy. Liposomes with enhanced circulation time are disclosed in U.S. Patent No. 5,013,556.

Another use of the present invention comprises incorporating an hVEGF antagonist into formed articles. Such articles can be used, for instance, in modulating endothelial cell growth and angiogenesis. In addition, tumor invasion and metastasis may be modulated with these articles.

An appropriate and effective dosage of antagonist will depend on the type of disease or condition to be treated, as defined herein, the severity and course of the disease or condition, whether the antagonists are administered for preventive or therapeutic purposes, previous therapy, the patient's clinical history and response to the antagonist, and the discretion of the attending physician. An effective dosage of antagonist will typically be that amount of antagonist administered to achieve the maximal of desired amount of inhibition of VEGF biological activity. The antagonist is suitably administered to the patient at one time or over a series of treatments.

The hVEGF antagonists are useful in the treatment of various neoplastic and non-neoplastic diseases and conditions. Neoplasms and related conditions that are amenable to treatment include breast carcinomas, lung carcinomas, gastric carcinomas, esophageal carcinomas, colorectal carcinomas, liver carcinomas, ovarian carcinomas, thecomas, arrhenoblastomas, cervical carcinomas, endometrial carcinoma, endometrial

hyperplasia, endometriosis, fibrosarcomas, choriocarcinoma, head and neck cancer, nasopharyngeal carcinoma, laryngeal carcinomas, hepatoblastoma, Kaposi's sarcoma, melanoma, skin carcinomas, hemangioma, cavernous hemangioma, hemangioblastoma, pancreas carcinomas, retinoblastoma, astrocytoma, glioblastoma, Schwannoma, oligodendroglioma, medulloblastoma, neuroblastomas, rhabdomyosarcoma, osteogenic sarcoma, leiomyosarcomas, urinary tract carcinomas, thyroid carcinomas, Wilm's tumor, renal cell carcinoma, prostate carcinoma, abnormal vascular proliferation associated with phakomatoses, and Meigs' syndrome.

In one embodiment, vascularization of tumors is attacked in combination therapy. One or more hVEGF antagonists are administered to tumor-bearing patients at therapeutically effective doses as determined for example by observing necrosis of the tumor or its metastatic foci, if any. This therapy is continued until such time as no further beneficial effect is observed or clinical examination shows no trace of the tumor or any metastatic foci. Other auxiliary agents such as tumor necrosis factor (TNF), alpha-, beta-, or gamma-interferon, anti-HER2 antibody, heregulin, anti-heregulin antibody, D-factor, interleukin-1 (IL-1), interleukin-2 (IL-2), granulocyte-macrophage colony stimulating factor (GM-CSF), or agents that promote microvascular coagulation in tumors, such as anti-protein C antibody, anti-protein S antibody, or C4b binding protein (see Esmon, et al., PCT Patent Publication No. WO 91/01753, published 21 February 1991), or heat or radiation.

Since the auxiliary agents will vary in their effectiveness it is desirable to compare their impact on the tumor by matrix screening in conventional fashion. The administration of hVEGF antagonist and, for instance, TNF, can be repeated until the desired clinical effect is achieved. Alternatively, the hVEGF antagonist(s) can be administered together with TNF and, optionally, auxiliary agent(s). In instances where solid tumors are found in the limbs or in other locations susceptible to isolation from the general circulation, the therapeutic agents described herein are administered to the isolated tumor or organ.

In other embodiments, a FGF or platelet-derived growth factor (PDGF) antagonist, such as an anti-FGF or an anti-PDGF neutralizing antibody, is administered to the patient in conjunction with the hVEGF antagonist. Treatment with hVEGF antagonists optimally may be suspended during periods of wound healing or desirable neovascularization.

Non-neoplastic conditions that are amenable to treatment include rheumatoid arthritis, psoriasis, atherosclerosis, diabetic and other retinopathies, retrolental fibroplasia, neovascular glaucoma, age-related macular degeneration, thyroid hyperplasias (including Grave's disease),
5 corneal and other tissue transplantation, chronic inflammation, lung inflammation, nephrotic syndrome, preeclampsia, ascites, pericardial effusion (such as that associated with pericarditis), and pleural effusion.

Age-related macular degeneration (AMD) is a leading cause of severe
10 visual loss in the elderly population. The exudative form of AMD is characterized by choroidal neovascularization and retinal pigment epithelial cell detachment. Because choroidal neovascularization is associated with a dramatic worsening in prognosis, the VEGF antagonists of the present invention are expected to be especially useful in reducing
15 the severity of AMD.

Other conditions that are amendable to treatment include edema. Herein, the term "edema" is used in a general sense and includes conditions in the body or accompanying stroke or head injury characterized by an increase in the extravascular tissue water content,
20 either due to increased free extracellular water alone, or in combination with increased intracellular water. The edema may be present in various tissues in the body. In particular, it is contemplated that the hVEGF antagonists may be employed to treat central nervous system (CNS) edema, including cerebral edema, typically characterized by an increase in brain
25 volume, as well as spinal cord or spinal canal edema or other conditions leading to increased intracranial pressure (such as local spinal cord injury). Increase in brain volume can be, for instance, the result of increased cerebral blood volume and/or increased tissue water content. The term "edema" used herein includes the pathological conditions
30 referred to in the art as vasogenic edema and cytotoxic edema. Typically, the condition referred to as vasogenic edema has been characterized as being associated with the disruption of the blood-brain barrier (BBB) while cytotoxic edema has been characterized as being associated with an intact BBB. Cerebral edema is described generally in
35 the review article, Hariri, Neurosurgical Intensive Care 5:687 (1994).

Edema in a mammal may result from or accompany a variety of pathological conditions or stimuli, including but not limited to, acute hypertension, meningitis, encephalitis, abscess, neoplastic diseases

(such as described above) (particularly solid tumors), trauma (such as head injury), hemorrhage, viral infection, cerebral malaria, stroke, radiation, multiple sclerosis, post cardiac arrest, birth asphyxia, glutamate toxicity, encephalopathy, hypoxia, ischemia and renal dialysis.

5 In particular, the invention contemplates therapy using the hVEGF antagonists to treat cerebral edema, including cerebral edema accompanying neoplasm(s) in the brain and cerebral edema accompanying stroke. In mammals having a neoplasm(s) in brain tissue, it is common for the mammal to develop or experience cerebral edema. It is
10 contemplated that the hVEGF antagonists of the present invention can be administered, alone or in combination with other therapies, like chemotherapy or radiation therapy administered to treat the brain neoplasm, to reduce or inhibit such edema in the brain.

It is also common for mammals having or having undergone stroke to
15 develop or experience cerebral edema. The term stroke in the present application is used in a general sense and includes the clinical conditions known to the skilled practitioner as ischemic stroke and hemorrhagic stroke. It is recognized within the art that stroke in a patient may be characterized or classified as various particular types of
20 stroke, depending for instance, upon the etiology or pathology of the interruption of blood flow, the types of cells or tissues affected, and the presence of blood extravasation into tissue (such as brain tissue). The different types of stroke that have been clinically characterized include but are not limited to, thrombotic stroke, embolic stroke,
25 hemodynamic stroke, lacunar stroke, and hemorrhagic strokes derived or resulting from intracerebral, subarachnoid, intraventricular, or subdural hemorrhage. The skilled medical practitioner will readily appreciate and understand the nature of such stroke conditions, and be able to detect and diagnosis the presence or symptoms of such conditions in patients.

30 The present inventive methods contemplate that the hVEGF antagonist molecules can be used in the treatment of all such stroke conditions, particularly to reduce or inhibit edema and protect against cell and tissue damage. The hVEGF antagonists can be administered as an acute treatment following stroke onset to reduce or inhibit for instance,
35 cerebral edema, thereby enhancing the mammal's recovery from the stroke.

The use of the hVEGF antagonists are beneficial in that the treatment may prevent or avoid having to perform surgery (like a craniotomy) on the mammal to reduce or alleviate intracranial pressure due to excess water

accumulation in brain tissues. It is also contemplated that upon reduction or prevention of such edema by the hVEGF antagonists, there will be a reduction (i.e., protective effect) in the amount of brain and neuronal tissue that can typically be damaged by intracranial pressure and edema.

Depending on the type and severity of the disease or condition being treated, about 1 µg/kg to 15 mg/kg of antagonist is an initial candidate dosage for administration to the patient, whether, for example, by one or more separate administrations, or by continuous infusion. A typical daily dosage might range from about 1 µg/kg to 100 mg/kg or more, depending on the factors mentioned above. For repeated administrations over several days or longer, depending on the condition, the treatment is repeated until a desired suppression of disease symptoms occurs. However, other dosage regimens may be useful. For instance, in the methods of treating cerebral edema or stroke, it may be desirable to administer the hVEGF antagonist(s) immediately upon detection or diagnosis in the patient, within several hours of injury or onset of stroke, or within 1 to 4 days thereafter. The desired administration protocol will typically be within the discretion of the medical practitioner. The progress of the hVEGF antagonist therapy is easily monitored by conventional techniques and assays, including, for example, radiographic techniques (in particular, magnetic resonance imaging, MRI) for neoplastic conditions and edema formation associated with trauma or stroke, or monitoring intracranial pressure for cerebral edema.

According to another embodiment of the invention, the effectiveness of the antagonist in preventing or treating a condition or disease may be improved by administering the antagonist serially or in combination with another agent that is effective for those purposes, such as tumor necrosis factor (TNF), an antibody capable of inhibiting or neutralizing the angiogenic activity of acidic or basic fibroblast growth factor (FGF) or hepatocyte growth factor (HGF), an antibody capable of inhibiting or neutralizing the coagulant activities of tissue factor, protein C, or protein S (see Esmon, et al., PCT Patent Publication No. WO 91/01753, published 21 February 1991), an antibody capable of binding to HER2 receptor (see Hudziak, et al., PCT Patent Publication No. WO 89/06692, published 27 July 1989), or one or more conventional therapeutic agents such as, for example, alkylating agents, folic acid antagonists, anti-metabolites of nucleic acid metabolism, antibiotics, pyrimidine analogs,

5-fluorouracil, cisplatin, purine nucleosides, amines, amino acids, triazol nucleosides, or corticosteroids. Such other agents may be present in the composition being administered or may be administered separately. Particularly in the treatment of edema or stroke, the antagonist may be administered serially or in combination with agents such as antiviral, antifungal or antiparasitic agents, antibiotics, thrombolytic agents (such as t-PA), osmotic therapy agents (e.g., mannitol), or steroids (like Decadron or prednisone). Use of such agents in combination with the antagonist will be within the ordinary skill of the medical practitioner, and of course, selection of such agents would depend, for instance, on the disease or condition being treated.

In a further method of treatment provided in the present application, it is contemplated that the hVEGF antagonist may be administered serially with hVEGF, particularly in the treatment of stroke. Upon diagnosis or detection of stroke, the hVEGF antagonist may be administered immediately or within approximately 1 to 4 days after onset of the stroke. It is believed that following completion of the administration of the antagonist to reduce or inhibit edema formation, it may be beneficial to administer to the patient an amount of hVEGF sufficient to stimulate or promote re-vascularization. Preferably, the hVEGF would be a recombinant form of hVEGF and would be administered in a pharmaceutically-acceptable carrier.

Other Uses

The anti-hVEGF antibodies of the invention also are useful as affinity purification agents. In this process, the antibodies against hVEGF are immobilized on a suitable support, such a Sephadex resin or filter paper, using methods well known in the art. The immobilized antibody then is contacted with a sample containing the hVEGF to be purified, and thereafter the support is washed with a suitable solvent that will remove substantially all the material in the sample except the hVEGF, which is bound to the immobilized antibody. Finally, the support is washed with another suitable solvent, such as glycine buffer, pH 5.0, that will release the hVEGF from the antibody.

The following examples are offered by way of illustration only and are not intended to limit the invention in any manner. All patent and literature citations in the specification are hereby incorporated by reference in their entirety.

EXAMPLES

Commercially available reagents referred to in the examples were used according to manufacturer's instructions unless otherwise indicated.

5 The source of those cells identified in the following examples, and throughout the specification, by ATCC accession numbers is the American Type Culture Collection, Manassas, Virginia.

EXAMPLE 1

Preparation of Anti- hVEGF Monoclonal Antibodies

10 To obtain hVEGF conjugated to keyhole limpet hemocyanin (KLH) for immunization, recombinant hVEGF (165 amino acids), Leung, et al., Science 246:1306 (1989), was mixed with KLH at a 4:1 ratio in the presence of 0.05% glutaraldehyde and the mixture was incubated at room temperature
15 for 3 hours with gentle stirring. The mixture then was dialyzed against phosphate buffered saline (PBS) at 4° C. overnight.

Balb/c mice were immunized four times every two weeks by intraperitoneal injections with 5 µg of hVEGF conjugated to 20 µg of KLH, and were boosted with the same dose of hVEGF conjugated to KLH four days
20 prior to cell fusion.

Spleen cells from the immunized mice were fused with P3X63Ag8U.1 myeloma cells, Yelton, et al., Curr. Top. Microbiol. Immunol. 81:1 (1978), using 35% polyethylene glycol (PEG) as described. Yarmush, et al., Proc. Nat. Acad. Sci. 77:2899 (1980). Hybridomas were selected in
25 HAT medium.

Supernatants from hybridoma cell cultures were screened for anti-hVEGF antibody production by an ELISA assay using hVEGF-coated microtiter plates. Antibody that was bound to hVEGF in each of the wells was determined using alkaline phosphatase-conjugated goat anti-mouse IgG
30 immunoglobulin and the chromogenic substrate p-nitrophenyl phosphate. Harlow & Lane, Antibodies: A Laboratory Manual, p.597 (Cold Spring Harbor Laboratory, 1988). Hybridoma cells thus determined to produce anti-hVEGF antibodies were subcloned by limiting dilution, and two of those clones, designated A4.6.1 and B2.6.2, were chosen for further studies.

EXAMPLE 2

Characterization of Anti-hVEGF Monoclonal AntibodiesA. Antigen Specificity

The binding specificities of the anti-hVEGF monoclonal antibodies produced by the A4.6.1 and B2.6.2 hybridomas were determined by ELISA. The monoclonal antibodies were added to the wells of microtiter plates that previously had been coated with hVEGF, FGF, HGF, or epidermal growth factor (EGF). Bound antibody was detected with peroxidase conjugated goat anti-mouse IgG immunoglobulins. The results of those assays confirmed that the monoclonal antibodies produced by the A4.6.1 and B2.6.2 hybridomas bind to hVEGF, but not detectably to those other protein growth factors.

B. Epitope Mapping

A competitive binding ELISA was used to determine whether the monoclonal antibodies produced by the A4.6.1 and B2.6.2 hybridomas bind to the same or different epitopes (sites) within hVEGF. Kim, et al., Infect. Immun. 57:944 (1989). Individual unlabeled anti-hVEGF monoclonal antibodies (A4.6.1 or B2.6.2) or irrelevant anti-HGF antibody (IgG1 isotype) were added to the wells of microtiter plates that previously had been coated with hVEGF. Biotinylated anti-hVEGF monoclonal antibodies (BIO-A4.6.1 or BIO-B2.6.2) were then added. The ratio of biotinylated antibody to unlabeled antibody was 1:1000. Binding of the biotinylated antibodies was visualized by the addition of avidin-conjugated peroxidase, followed by o-phenylenediamine dihydrochloride and hydrogen peroxide. The color reaction, indicating the amount of biotinylated antibody bound, was determined by measuring the optical density (O.D) at 495 nm wavelength.

As shown in Figure 1, in each case, the binding of the biotinylated anti-hVEGF antibody was inhibited by the corresponding unlabeled antibody, but not by the other unlabeled anti-hVEGF antibody or the anti-HGF antibody. These results indicate that the monoclonal antibodies produced by the A4.6.1 and B2.6.2 hybridomas bind to different epitopes within hVEGF.

C. Isotyping

The isotypes of the anti-hVEGF monoclonal antibodies produced by the A4.6.1 and B2.6.2 hybridomas were determined by ELISA. Samples of

culture medium (supernatant) in which each of the hybridomas was growing were added to the wells of microtiter plates that had previously been coated with hVEGF. The captured anti-hVEGF monoclonal antibodies were incubated with different isotype-specific alkaline phosphatase-conjugated goat anti-mouse immunoglobulins, and the binding of the conjugated antibodies to the anti-hVEGF monoclonal antibodies was determined by the addition of p-nitrophenyl phosphate. The color reaction was measured at 405 nm with an ELISA plate reader.

By that method, the isotype of the monoclonal antibodies produced by both the A4.6.1 and B2.6.2 hybridomas was determined to be IgG1.

D. Binding Affinity

The affinities of the anti-hVEGF monoclonal antibodies produced by the A4.6.1 and B2.6.2 hybridomas for hVEGF were determined by a competitive binding assays. A predetermined sub-optimal concentration of monoclonal antibody was added to samples containing 20,000 - 40,000 cpm ^{125}I -hVEGF (1 - 2 ng) and various known amounts of unlabeled hVEGF (1 - 1000 ng). After 1 hour at room temperature, 100 μl of goat anti-mouse Ig antisera (Pel-Freez, Rogers, AR USA) were added, and the mixtures were incubated another hour at room temperature. Complexes of antibody and bound protein (immune complexes) were precipitated by the addition of 500 μl of 6% polyethylene glycol (PEG, mol. wt. 8000) at 4° C., followed by centrifugation at 2000 x G. for 20 min. at 4° C. The amount of ^{125}I -hVEGF bound to the anti-hVEGF monoclonal antibody in each sample was determined by counting the pelleted material in a gamma counter.

Affinity constants were calculated from the data by Scatchard analysis. The affinity of the anti-hVEGF monoclonal antibody produced by the A4.6.1 hybridoma was calculated to be 1.2×10^9 liters/mole. The affinity of the anti-hVEGF monoclonal antibody produced by the B2.6.2 hybridoma was calculated to be 2.5×10^9 liters/mole.

E. Inhibition of hVEGF Mitogenic Activity

Bovine adrenal cortex capillary endothelial (ACE) cells, Ferrara, *et al.*, Proc. Nat. Acad. Sci. 84:5773 (1987), were seeded at a density of 10^4 cells/ml in 12 multiwell plates, and 2.5 ng/ml hVEGF was added to each well in the presence or absence of various concentrations of the anti-hVEGF monoclonal antibodies produced by the A4.6.1 or B2.6.2 hybridomas, or an irrelevant anti-HGF monoclonal antibody. After

culturing 5 days, the cells in each well were counted in a Coulter counter. As a control, ACE cells were cultured in the absence of added hVEGF.

As shown in Figure 2, both of the anti-hVEGF monoclonal antibodies inhibited the ability of the added hVEGF to support the growth or survival of the bovine ACE cells. The monoclonal antibody produced by the A4.6.1 hybridoma completely inhibited the mitogenic activity of hVEGF (greater than about 90% inhibition), whereas the monoclonal antibody produced by the B2.6.2 hybridoma only partially inhibited the mitogenic activity of hVEGF.

F. Inhibition of hVEGF Binding

Bovine ACE cells were seeded at a density of 2.5×10^4 cells/0.5 ml/well in 24 well microtiter plates in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% calf serum, 2 mM glutamine, and 1 ng/ml basic fibroblast growth factor. After culturing overnight, the cells were washed once in binding buffer (equal volumes of DMEM and F12 medium plus 25 mM HEPES and 1% bovine serum albumin) at 4° C.

12,000 cpm 125 I-hVEGF (approx. 5×10^4 cpm/ng/ml) was preincubated for 30 minutes with 5 µg of the anti-hVEGF monoclonal antibody produced by the A4.6.1, B2.6.2, or A2.6.1 hybridoma (250 µl total volume), and thereafter the mixtures were added to the bovine ACE cells in the microtiter plates. After incubating the cells for 3 hours at 4° C., the cells were washed 3 times with binding buffer at 4° C., solubilized by the addition of 0.5 ml 0.2 N. NaOH, and counted in a gamma counter.

As shown in Figure 3 (upper), the anti-hVEGF monoclonal antibodies produced by the A4.6.1 and B2.6.2 hybridomas inhibited the binding of hVEGF to the bovine ACE cells. In contrast, the anti-hVEGF monoclonal antibody produced by the A2.6.1 hybridoma had no apparent effect on the binding of hVEGF to the bovine ACE cells. Consistent with the results obtained in the cell proliferation assay described above, the monoclonal antibody produced by the A4.6.1 hybridoma inhibited the binding of hVEGF to a greater extent than the monoclonal antibody produced by the B2.6.2 hybridoma.

As shown in Figure 3 (lower), the monoclonal antibody produced by the A4.6.1 hybridoma completely inhibited the binding of hVEGF to the bovine ACE cells at a 1:250 molar ratio of hVEGF to antibody.

G. Cross-reactivity with other VEGF isoforms

To determine whether the anti-hVEGF monoclonal antibody produced by the A4.6.1 hybridoma is reactive with the 121- and 189-amino acid forms of hVEGF, the antibody was assayed for its ability to immunoprecipitate those polypeptides.

Human 293 cells were transfected with vectors comprising the nucleotide coding sequence of the 121- and 189-amino acid hVEGF polypeptides, as described. Leung, et al., Science 246:1306 (1989). Two days after transfection, the cells were transferred to medium lacking cysteine and methionine. The cells were incubated 30 minutes in that medium, then 100 μ Ci/ml of each 35 S-methionine and 35 S-cysteine were added to the medium, and the cells were incubated another two hours. The labeling was chased by transferring the cells to serum free medium and incubating three hours. The cell culture media were collected, and the cells were lysed by incubating for 30 minutes in lysis buffer (150 mM NaCl, 1% NP40, 0.5% deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 50 mM Tris, pH 8.0). Cell debris was removed from the lysates by centrifugation at 200 x G. for 30 minutes.

500 μ l samples of cell culture media and cell lysates were incubated with 2 μ l of A4.6.1 hybridoma antibody (2.4 mg/ml) for 1 hour at 4° C., and then were incubated with 5 μ l of rabbit anti-mouse IgG immunoglobulin for 1 hour at 4° C. Immune complexes of 35 S-labeled hVEGF and anti-hVEGF monoclonal antibody were precipitated with protein-A Sepharose (Pharmacia), then subjected to SDS - 12% polyacrylamide gel electrophoresis under reducing conditions. The gel was exposed to x-ray film for analysis of the immunoprecipitated, radiolabeled proteins by autoradiography.

The results of that analysis indicated that the anti-hVEGF monoclonal antibody produced by the A4.6.1 hybridoma was cross-reactive with both the 121- and 189-amino acid forms of hVEGF.

EXAMPLE 3Preparation of VEGF Receptor - IgG Fusion Proteins

A.

The nucleotide and amino acid coding sequences of the flt hVEGF receptor are disclosed in Shibuya, et al., Oncogene 5:519-524 (1990).

The coding sequence of the entire extracellular domain of the flt hVEGF receptor was fused to the coding sequence of human IgG1 heavy chain in a two-step process.

Site-directed mutagenesis was used to introduce a BstBI restriction
5 into DNA encoding flt at a site 5' to the codon for amino acid 759 of
flt, and to convert the unique BstEII restriction site in plasmid pBSSK'
FC, Bennett, et al., J. Biol. Chem. 266:23060-23067 (1991), to a BstBI
site. The modified plasmid was digested with EcoRI and BstBI and the
10 resulting large fragment of plasmid DNA was ligated together with an
EcoRI-BstBI fragment of the flt DNA encoding the extracellular domain
(amino acids 1-758) of the flt hVEGF receptor.

The resulting construct was digested with ClaI and NotI to generate
an approximately 3.3 kb fragment, which is then inserted into the
multiple cloning site of the mammalian expression vector pHEBO2 (Leung,
15 et al., Neuron 8:1045 (1992)) by ligation. The ends of 3.3. kb fragment
are modified, for example, by the addition of linkers, to obtain
insertion of the fragment into the vector in the correct orientation for
expression.

Mammalian host cells (for example, CEN4 cells (Leung, et al. supra)
20 are transfected with the pHEBO2 plasmid containing the flt insert by
electroporation. Transfected cells are cultured in medium containing
about 10% fetal bovine serum, 2 mM glutamine, and antibiotics, and at
about 75% confluency are transferred to serum free medium. Medium is
conditioned for 3-4 days prior to collection, and the flt-IgG fusion
25 protein is purified from the conditioned medium by chromatography on a
protein-A affinity matrix essentially as described in Bennett, et al., J.
Biol. Chem. 266:23060-23067 (1991).

B.

A human flt-IgG (referred to as hflt(1-3)-IgG) cDNA was constructed
30 as described in Davis-Smyth et al., EMBO J. 15:4919-4927 (1996). This
truncated receptor form included only the first three immunoglobulin-like
domains of human flt fused to a Fc-IgG. See Ferrara et al., Nature
Medicine 4:336 (1998).

A murine flt-IgG (referred to as mflt(1-3)-IgG) was constructed by
35 PCR amplification of mouse 17-day embryo cDNA (Clontech, Palo Alto, CA)
using primers described in Ferrara et al., supra. The design of the 3'
PCR primer ensured that the expression of the mflt-1(1-3) was in frame
with a murine IgG2b Fc clone. The resulting 1-kb fragment was first

cloned into a TA cloning vector (Invitrogen, San Diego, CA) as a ClaI-BstEII fragment. This fragment was ligated to the 5' end of murine IgG2b Fc in a pRK vector. This plasmid enabled the expression of mflt(1-3)-IgG fusion protein when transfected into mammalian cells.

5 For expression in CHO cells, the cDNAs were subcloned into a dicistronic vector that links the expression of the marker dihydrofolate reductase to the expression of the flt derived fusion protein. See, Lucas et al., Nucleic Acid Res. 24:1774-1779 (1996). Plasmids were introduced into DP12 cells, a derivative of the CHO-K1DUXB11 cell line
10 developed by L. Chasin (Columbia University, New York) via lipofection and selected for growth in glycine-hypoxanthine-thymidine (G-H-T)-free medium. Chisholm et al., DNA Cloning 4:A Practical Approach, Mammalian Systems (eds. Glover & Hames) pp. 1-39 (Oxford Press, 1995). Clones from the first round of selection were subsequently plated at increasing
15 concentrations of methotrexate. Clones were then screened for production by ELISA for the human or murine Fc. Clones that displayed the highest production were adapted to suspension culture, and serum-free cultures were harvested and purified by protein A-Sepharose. Protein concentrations were determined by amino acid analysis. The endotoxin
20 content of the final purified material did not exceed 0.5 eu/mg.

As described in Ferrara et al., supra, both the murine flt(1-3)-IgG fusion protein and the human flt(1-3)-IgG fusion protein were active in inhibiting bioactivity of VEGF in the tested rodent model.

25 EXAMPLE 4

Inhibition of Tumor Growth with hVEGF Antagonists

Various human tumor cell lines growing in culture were assayed for production of hVEGF by ELISA. Ovary, lung, colon, gastric, breast, and brain tumor cell lines were found to produce hVEGF. Three cell lines
30 that produced hVEGF, NEG 55 (also referred to as G55) (human glioma cell line obtained from Dr. M. Westphal, Department of Neurosurgery, University Hospital Eppendorf, Hamburg, Germany, also referred to as G55), A-673 (human rhabdomyosarcoma cell line obtained from the American Type Culture Collection (ATCC), as cell line number CRL 1598), and SK-LMS-1
35 (leiomyosarcoma cell line obtained from the ATCC as cell line number HTB 88), were used for further studies.

Six to ten week old female Beige/nude mice (Charles River Laboratory, Wilmington, Massachusetts USA) were injected subcutaneously with $1 - 5 \times 10^6$ tumor cells in 100-200 μ l PBS. At various times after tumor growth was established, mice were injected intraperitoneally once or twice per week with various doses of A4.6.1 anti-hVEGF monoclonal antibody, an irrelevant anti-gp120 monoclonal antibody (5B6), or PBS. Tumor size was measured every week, and at the conclusion of the study the tumors were excised and weighed.

The effect of various amounts of A4.6.1 anti-hVEGF monoclonal antibody on the growth of NEG 55 tumors in mice is shown in Figures 4 and 5. Figure 4 shows that mice treated with 25 μ g or 100 μ g of A4.6.1 anti-hVEGF monoclonal antibody beginning one week after inoculation of NEG 55 cells had a substantially reduced rate of tumor growth as compared to mice treated with either irrelevant antibody or PBS. Figure 5 shows that five weeks after inoculation of the NEG 55 cells, the size of the tumors in mice treated with A4.6.1 anti-hVEGF antibody was about 50% (in the case of mice treated with 25 μ g dosages of the antibody) to 85% (in the case of mice treated with 100 μ g dosages of the antibody) less than the size of tumors in mice treated with irrelevant antibody or PBS.

The effect of A4.6.1 anti-hVEGF monoclonal antibody treatment on the growth of SK-LMS-1 tumors in mice is shown in Figure 6. Five weeks after inoculation of the SK-LMS-1 cells, the average size of tumors in mice treated with the A4.6.1 anti-hVEGF antibody was about 75% less than the size of tumors in mice treated with irrelevant antibody or PBS.

The effect of A4.6.1 anti-hVEGF monoclonal antibody treatment on the growth of A673 tumors in mice is shown in Figure 7. Four weeks after inoculation of the A673 cells, the average size of tumors in mice treated with A4.6.1 anti-hVEGF antibody was about 60% (in the case of mice treated with 10 μ g dosages of the antibody) to greater than 90% (in the case of mice treated with 50-400 μ g dosages of the antibody) less than the size of tumors in mice treated with irrelevant antibody or PBS.

EXAMPLE 5

Analysis of the Direct Effect of Anti-hVEGF Antibody
on Tumor Cells Growing in Culture

NEG55 human glioblastoma cells or A673 rhabdomyosarcoma cells were
5 seeded at a density of 7×10^3 cells/well in multiwell plates (12
wells/plate) in F12/DMEM medium containing 10% fetal calf serum, 2mM
glutamine, and antibiotics. A4.6.1 anti-hVEGF antibody then was added to
the cell cultures to a final concentration of 0 - 20.0 μ g antibody/ml.
After five days, the cells growing in the wells were dissociated by
10 exposure to trypsin and counted in a Coulter counter.

Figures 8 and 9 show the results of those studies. As is apparent,
the A4.6.1 anti-hVEGF antibody did not have any significant effect on the
growth of the NEG55 or A673 cells in culture. These results indicate
that the A4.6.1 anti-hVEGF antibody is not cytotoxic, and strongly
15 suggest that the observed anti-tumor effects of the antibody are due to
its inhibition of VEGF-mediated neovascularization.

EXAMPLE 6

Effect of Anti-hVEGF Antibody on Endothelial Cell Chemotaxis

20 Chemotaxis of endothelial cells and others cells, including
monocytes and lymphocytes, play an important role in the pathogenesis of
rheumatoid arthritis. Endothelial cell migration and proliferation
accompany the angiogenesis that occurs in the rheumatoid synovium.
Vascularized tissue (pannus) invades and destroys the articular
25 cartilage.

To determine whether hVEGF antagonists interfere with this process,
we assayed the effect of the A4.6.1 anti-hVEGF antibody on endothelial
cell chemotaxis stimulated by synovial fluid from patients having
rheumatoid arthritis. As a control, we also assayed the effect of the
30 A4.6.1 anti-hVEGF antibody on endothelial cell chemotaxis stimulated by
synovial fluid from patients having osteoarthritis (the angiogenesis that
occurs in rheumatoid arthritis does not occur in osteoarthritis).

Endothelial cell chemotaxis was assayed using modified Boyden
chambers according to established procedures. Thompson, et al., Cancer
35 Res. 51:2670 (1991); Phillips, et al., Proc. Exp. Biol. Med. 197:458
(1991). About 10^4 human umbilical vein endothelial cells were allowed to
adhere to gelatin-coated filters (0.8 micron pore size) in 48-well

multiwell microchambers in culture medium containing 0.1% fetal bovine serum. After about two hours, the chambers were inverted and test samples (rheumatoid arthritis synovial fluid, osteoarthritis synovial fluid, basic FGF (bFGF) (to a final concentration of 1 µg/ml), or PBS) and A4.6.1 anti-hVEGF antibody (to a final concentration of 10 µg/ml) were added to the wells. After two to four hours, cells that had migrated were stained and counted.

Figure 10 shows the averaged results of those studies. The values shown in the column labeled "Syn. Fluid" and shown at the bottom of the page for the controls are the average number of endothelial cells that migrated in the presence of synovial fluid, bFGF, or PBS alone. The values in the column labeled "Syn. Fluid + mAB VEGF" are the average number of endothelial cells that migrated in the presence of synovial fluid plus added A4.6.1 anti-hVEGF antibody. The values in the column labeled "% Suppression" indicate the percentage reduction in synovial fluid-induced endothelial cell migration resulting from the addition of anti-hVEGF antibody. As indicated, the anti-hVEGF antibody significantly inhibited the ability of rheumatoid arthritis synovial fluid (53.40 average percentage inhibition), but not osteoarthritis synovial fluid (13.64 average percentage inhibition), to induce endothelial cell migration.

EXAMPLE 7

Effect of VEGF Antagonist on Cerebral Edema

An *in vivo* assay was conducted to determine the effects of a flt-IgG antagonist on cerebral edema. Loss of BBB integrity and the formation of cerebral edema often occurs in the pathogenesis of cerebral infarction. It is believed that breakdown of the BBB in ischemic stroke occurs predominantly after the first 24 hours of stroke onset. Further, it is believed that the beneficial effects of prompt and adequate restoration of blood flow following an acute ischemic event may be undermined by reperfusion injury to the cerebral microvasculature comprising the BBB, contributing to the formation of cerebral edema. Klatzo et al. Eds., Brain Edema, Tokyo, Springer (1984), pp. 1-5. The *in vivo* assay described below was designed to reflect these aspects of the clinical condition.

Focal cortical ischemia was induced in mouse brain by the occlusion of the middle cerebral artery (MCA) using the techniques previously described by Chen *et al.*, Stroke 17:738-743 (1986). The mice (C57BL-6J; 18-25 grams) were anesthetized with 1.5% isoflurane in oxygen. The right MCA was exposed via a craniotomy and ligated with a 11-0 suture. The ipsilateral common carotid artery was also occluded for the ischemic period. The vessels remained occluded for 45 minutes. Prior to surgery, the animals were randomly divided into two groups and either murine *flt*-IgG (as described in Example 3B above; also described in Ferrara *et al.*, Nature Medicine 4:336 (1998)) or an irrelevant control murine anti-GP120 antibody belonging to the same isotype as the Fc in the *flt*-IgG [Ferrara *et al.*, *supra*] was administered intraperitoneally at a dose of 10 mg/kg at 12 hours prior to surgery, at the time of reperfusion and again at 1 and 2 days following surgery. The degree of edema formation was assessed by T2 weighted MR imaging 24 hours following the onset of ischemia. The eventual size of the infarction was assessed 8-12 weeks later using high resolution anatomical MRI. A subset of animals (n=12) were taken for verification of infarction size using conventional histology techniques.

As shown in Fig. 11, administration of *flt*-Ig caused a significant reduction in the volume of cerebral edema as defined by the region of hyperintensity on the T2-weighted MRI scan acquired 1 day following onset of ischemia (27% reduction, p=0.01 Student's t-test, n=15 and 16 in control and treatment groups, respectively). Representative T2-weighted MR images showing the appearance of cortical edema as a region of high signal intensity compared to the contralateral side is shown in Fig. 12.

In this model, progression of ischemic damage leads to loss of cortical tissue and cavitation. The ultimate infarction volume can, therefore, be estimated from high resolution anatomical images by delineating the amount of unaffected cortex and comparing it to the contralateral hemisphere. As shown in Fig. 13, the size of the cortical infarction is significantly reduced by the administration of *flt*-IgG measured 8-12 weeks later (26% reduction in infarct size, p=0.009 Student's t-test, n=11 and 14 in control and treatment groups, respectively). There was a good correlation between the infarct volume measured by MRI and that determined using conventional histology ($R^2=.633$). Accordingly, the treated animals exhibited a reduction in development of cerebral edema, which may further provide enhanced neuroprotection. These results

indicate that inhibition of the biological activity of VEGF can reduce ischemic-reperfusion related brain edema and injury.

What is claimed is:

1. A method of treating a mammal having edema comprising administering to said mammal an effective amount of hVEGF antagonist.
- 5 2. The method of claim 1 wherein said edema comprises cerebral edema.
3. The method of claim 1 wherein said mammal is a human further having a neoplastic disease.
- 10 4. The method of claim 3 wherein said neoplastic disease comprises a brain tumor.
5. The method of claim 4 wherein said hVEGF antagonist is administered to said mammal serially or in combination with chemotherapy or radiation
15 therapy.
6. The method of claim 1 wherein said mammal is a human further having or having undergone a stroke.
- 20 7. The method of claim 1 wherein said hVEGF antagonist comprises an anti-hVEGF antibody.
8. The method of claim 7 wherein said anti-hVEGF antibody comprises a chimeric antibody.
- 25 9. The method of claim 7 wherein said anti-hVEGF antibody comprises a humanized antibody.
10. The method of claim 7 wherein said antibody comprises a monoclonal
30 antibody.
11. The method of claim 1 wherein said hVEGF antagonist comprises a hVEGF receptor fusion protein.
- 35 12. The method of claim 11 wherein said hVEGF receptor fusion protein comprises an extracellular domain sequence of a hVEGF receptor fused to an immunoglobulin.

13. The method of claim 12 wherein said hVEGF receptor fusion protein comprises a flt-IgG fusion protein.

5 14. A method of treating a mammal having or having undergone a stroke, comprising administering to said mammal an effective amount of hVEGF antagonist.

15. The method of claim 14 wherein said hVEGF antagonist comprises an
10 anti-hVEGF antibody.

16. The method of claim 15 wherein said anti-hVEGF antibody comprises a chimeric antibody.

15 17. The method of claim 15 wherein said anti-hVEGF antibody comprises a humanized antibody.

18. The method of claim 15 wherein said antibody comprises a monoclonal
20 antibody.

19. The method of claim 14 wherein said hVEGF antagonist comprises a hVEGF receptor fusion protein.

20. The method of claim 19 wherein said hVEGF receptor fusion protein
25 comprises an extracellular domain sequence of a hVEGF receptor fused to an immunoglobulin.

21. The method of claim 20 wherein said hVEGF receptor fusion protein
30 comprises a flt-IgG fusion protein.

22. A method of treating a mammal having cerebral edema comprising
administering to said mammal an effective amount of hVEGF antagonist.

23. The method of claim 22 wherein said hVEGF antagonist comprises an
35 anti-hVEGF antibody.

24. The method of claim 23 wherein said anti-hVEGF antibody comprises a chimeric antibody.

25. The method of claim 23 wherein said anti-hVEGF antibody comprises a
5 humanized antibody.

26. The method of claim 23 wherein said antibody comprises a monoclonal antibody.

10 27. The method of claim 22 wherein said hVEGF antagonist comprises a hVEGF receptor fusion protein.

28. The method of claim 27 wherein said hVEGF receptor fusion protein
comprises an extracellular domain sequence of a hVEGF receptor fused to
15 an immunoglobulin.

29. The method of claim 28 wherein said hVEGF receptor fusion protein
comprises a flt-IgG fusion protein.

20

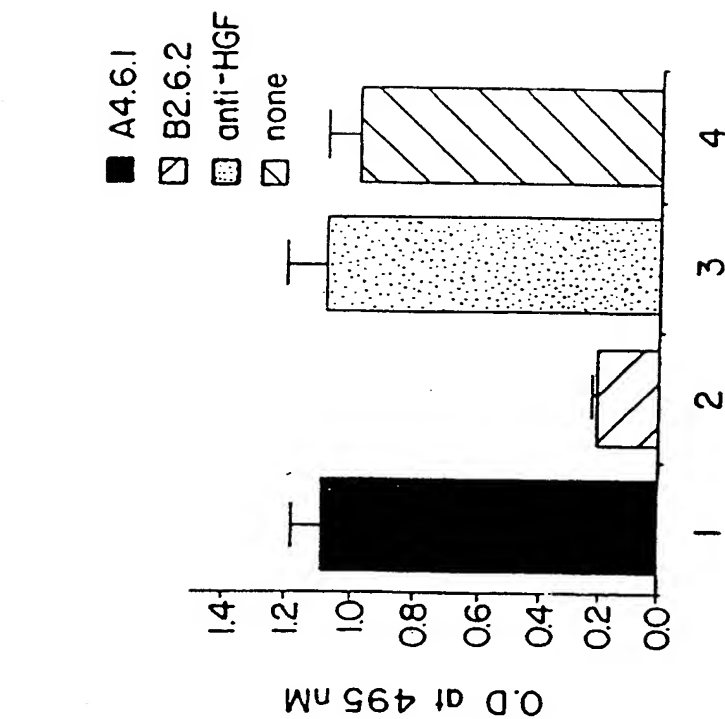


FIG. 1a

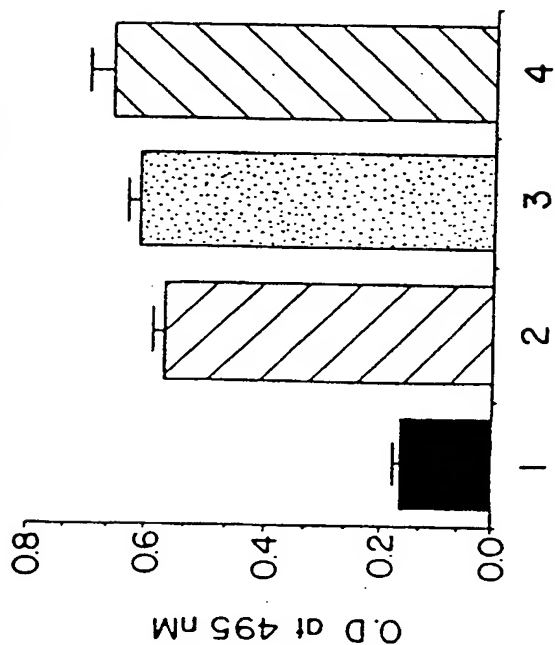


FIG. 1b

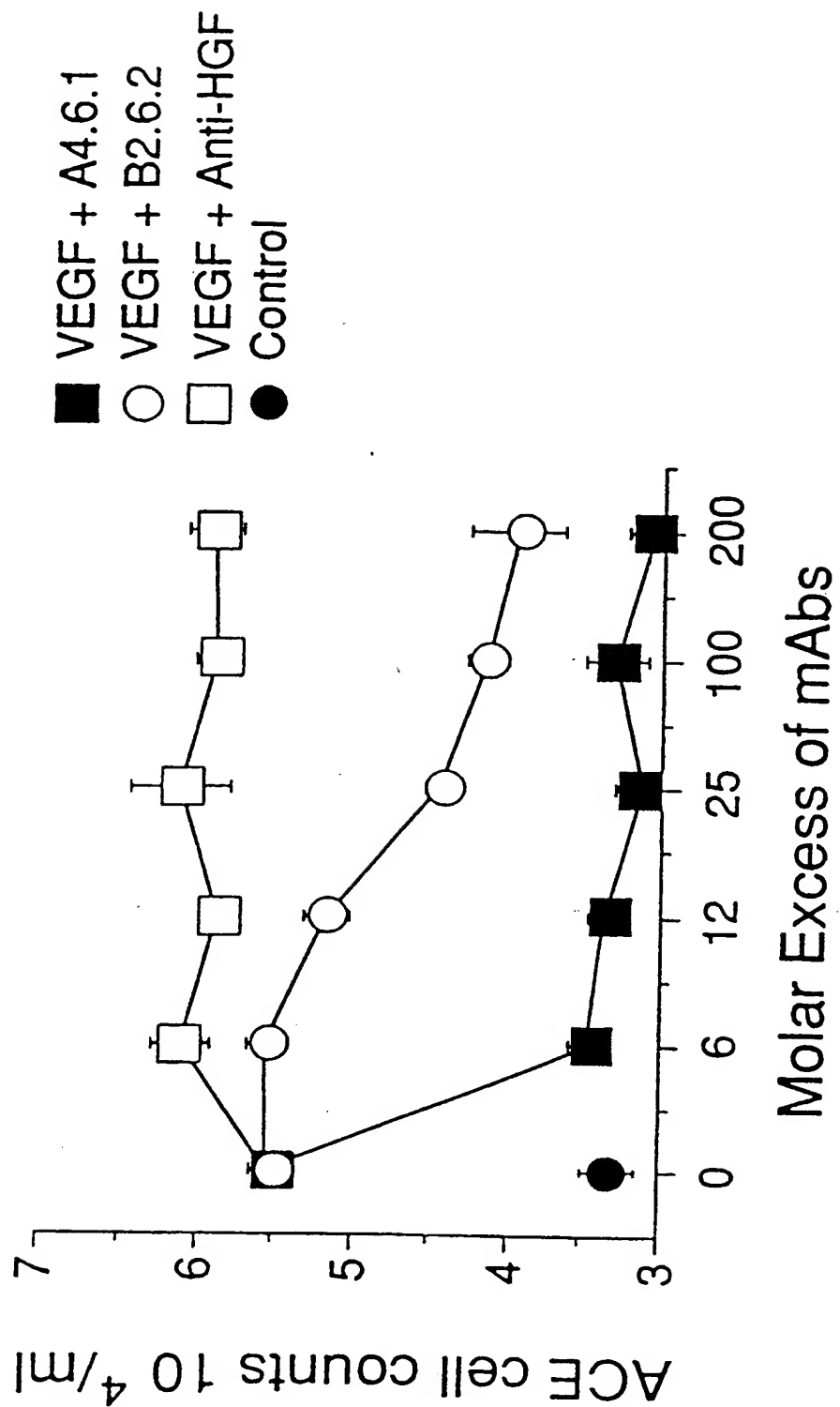


FIG. 2

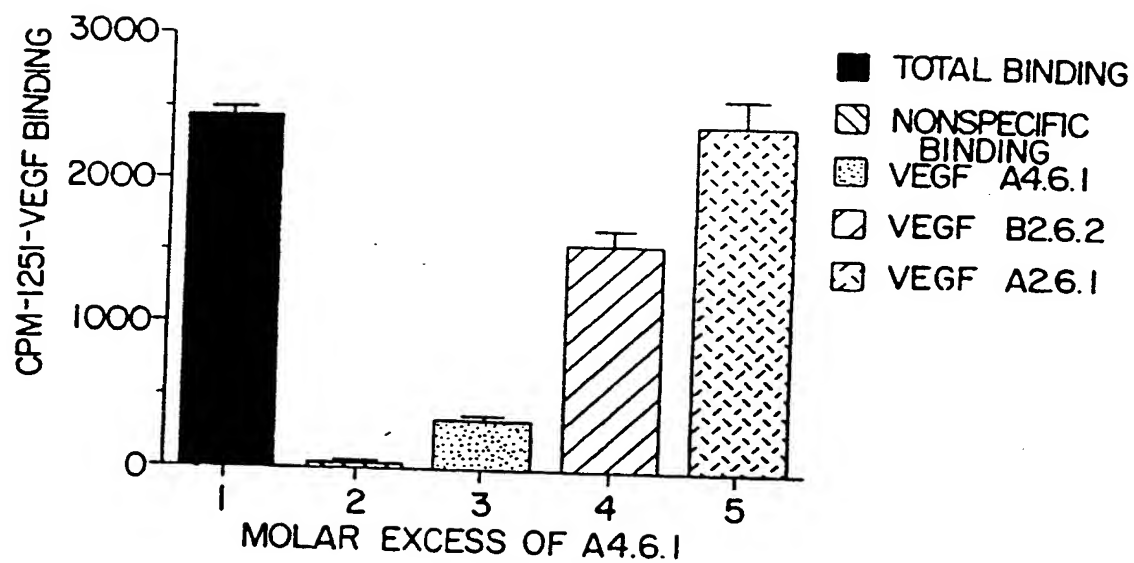


FIG. 3a

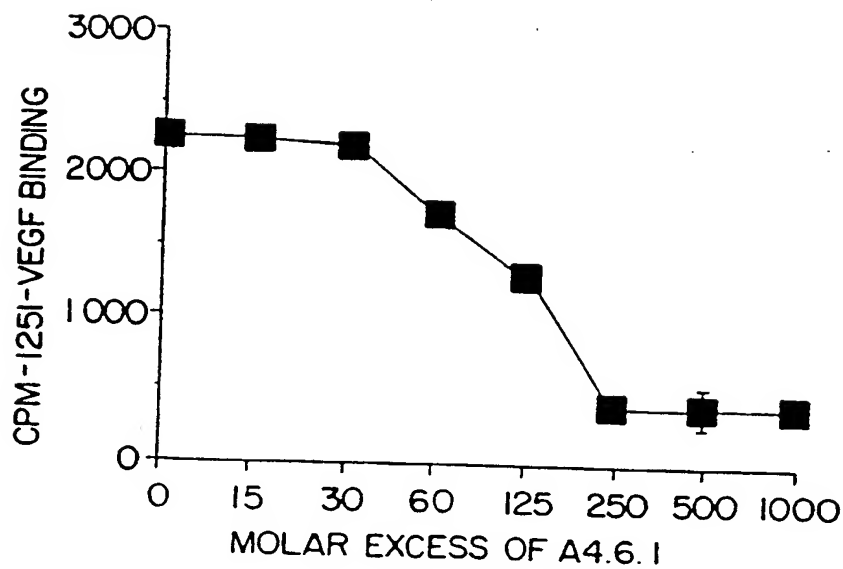
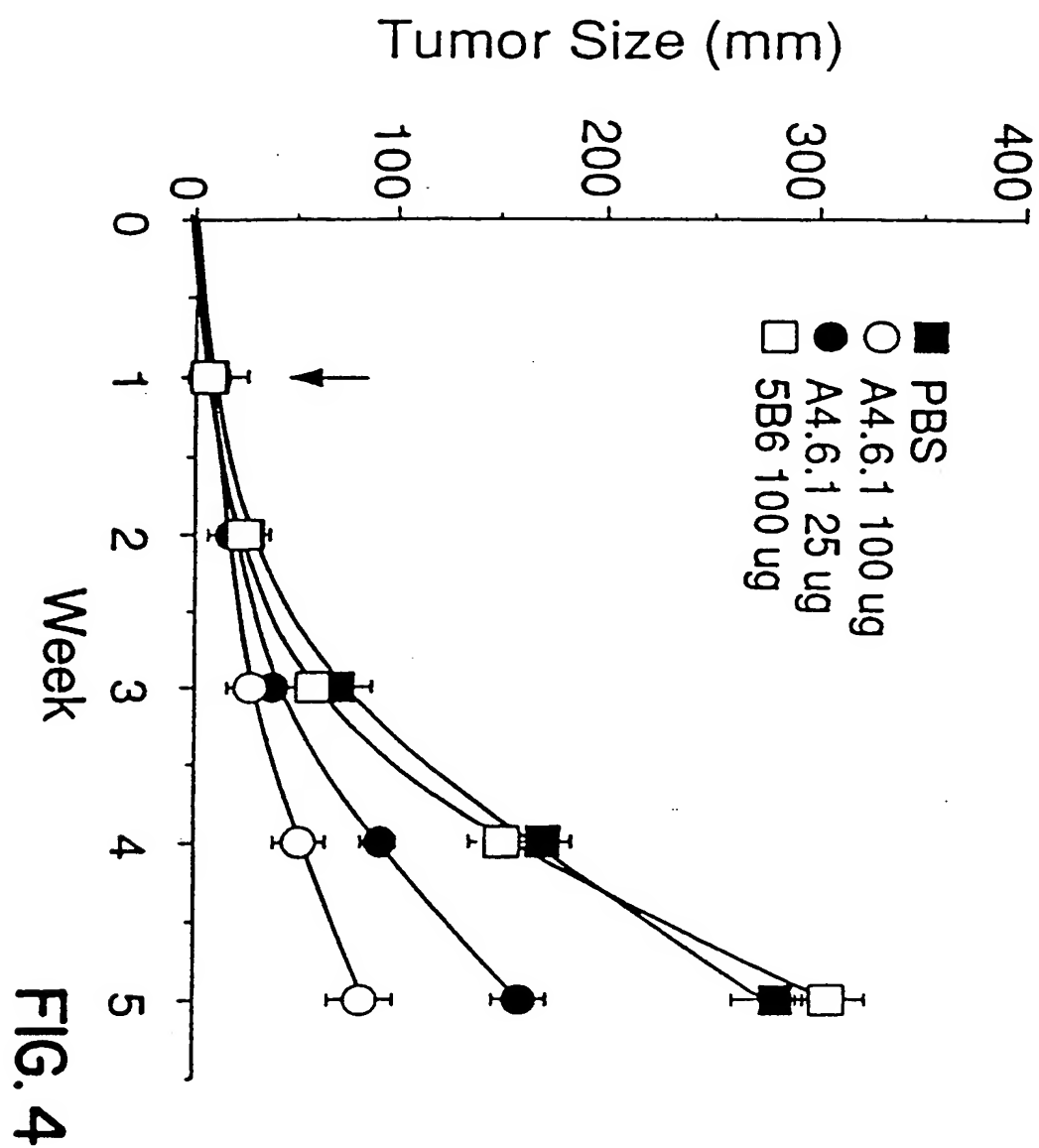


FIG. 3b



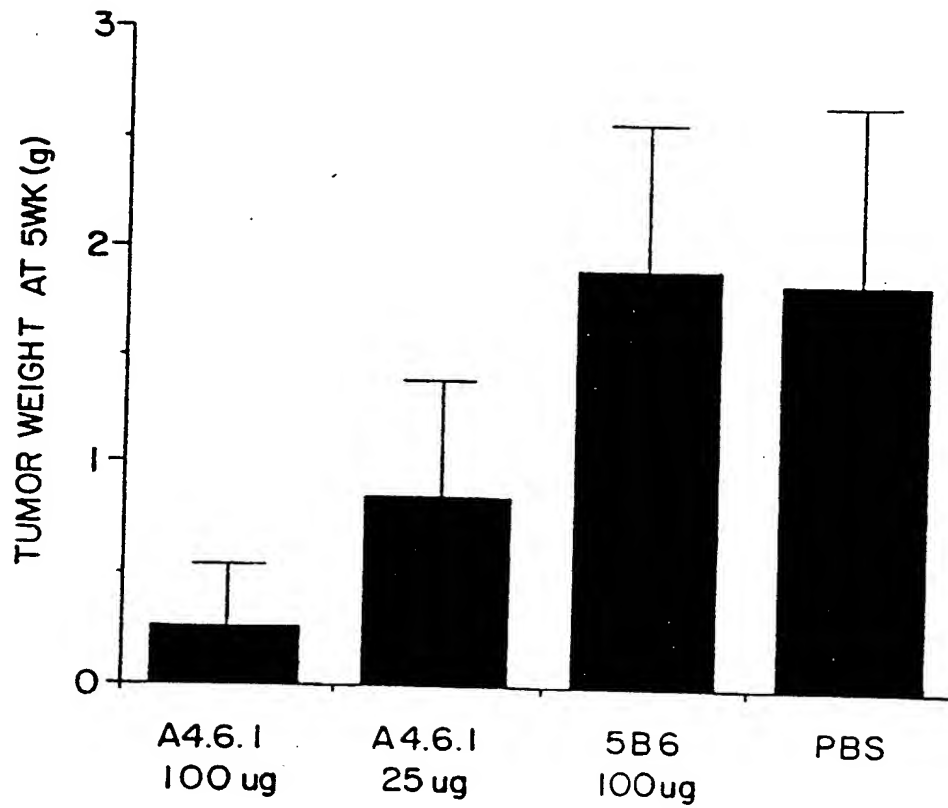


FIG. 5

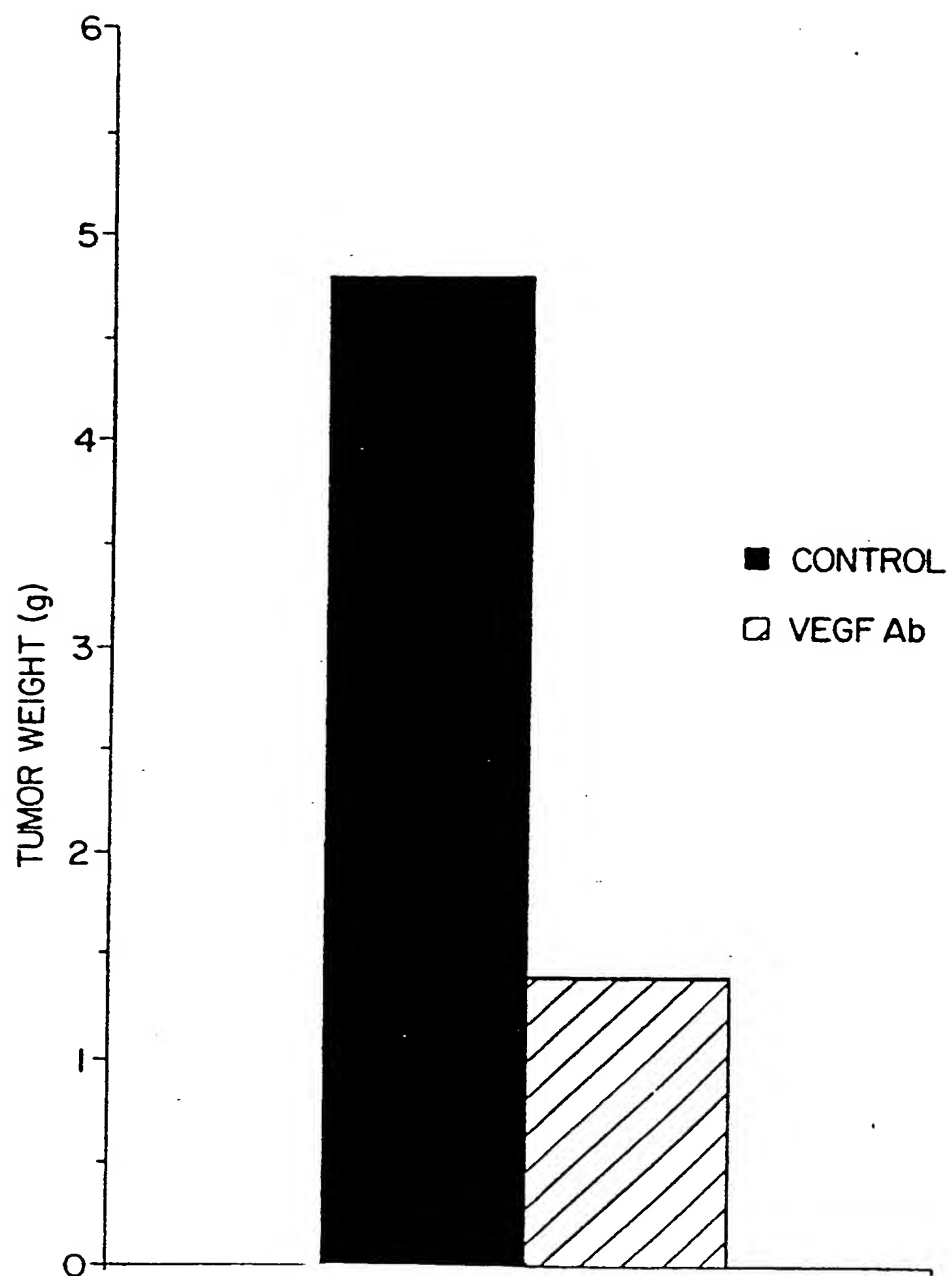


FIG. 6

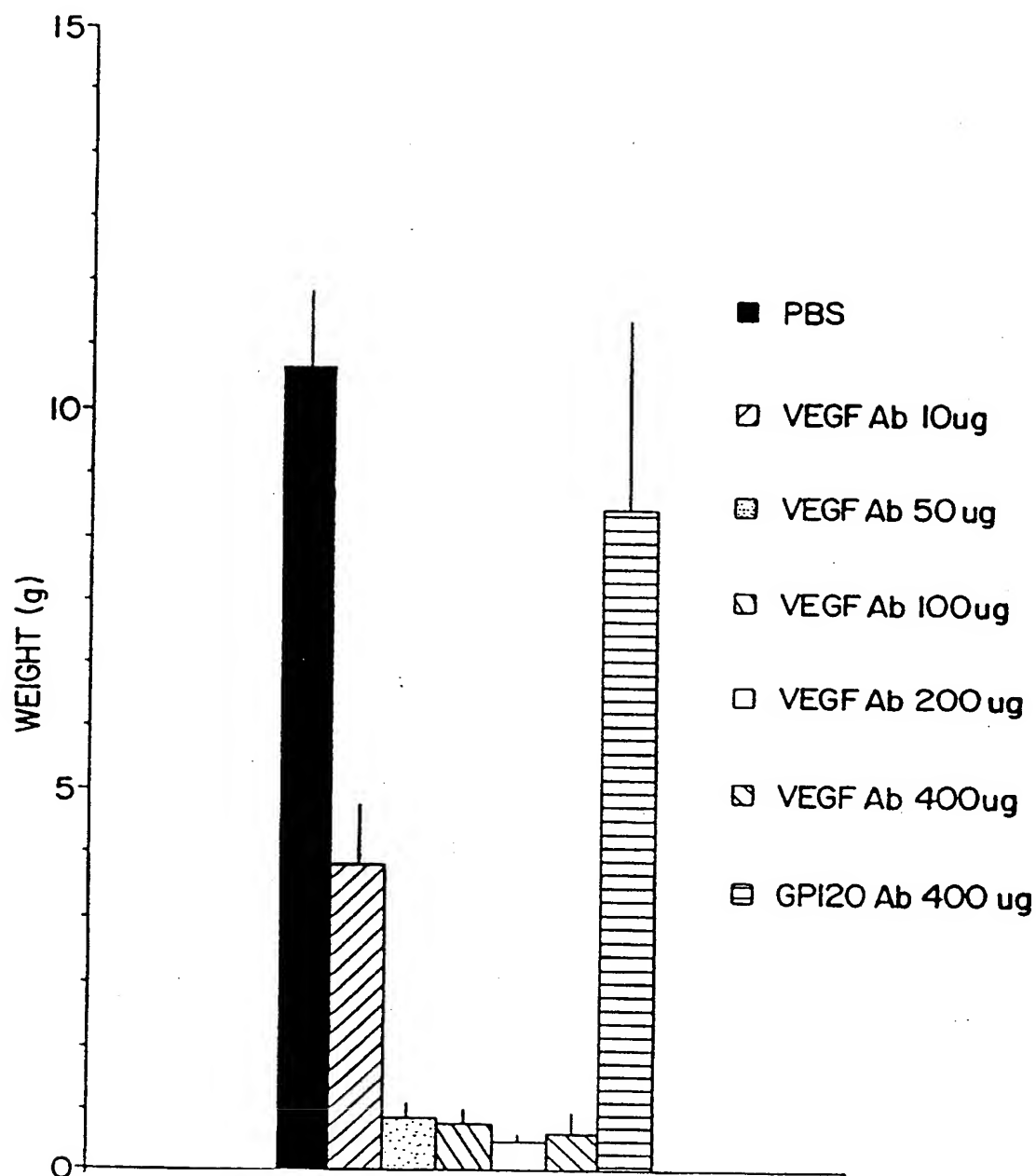


FIG. 7

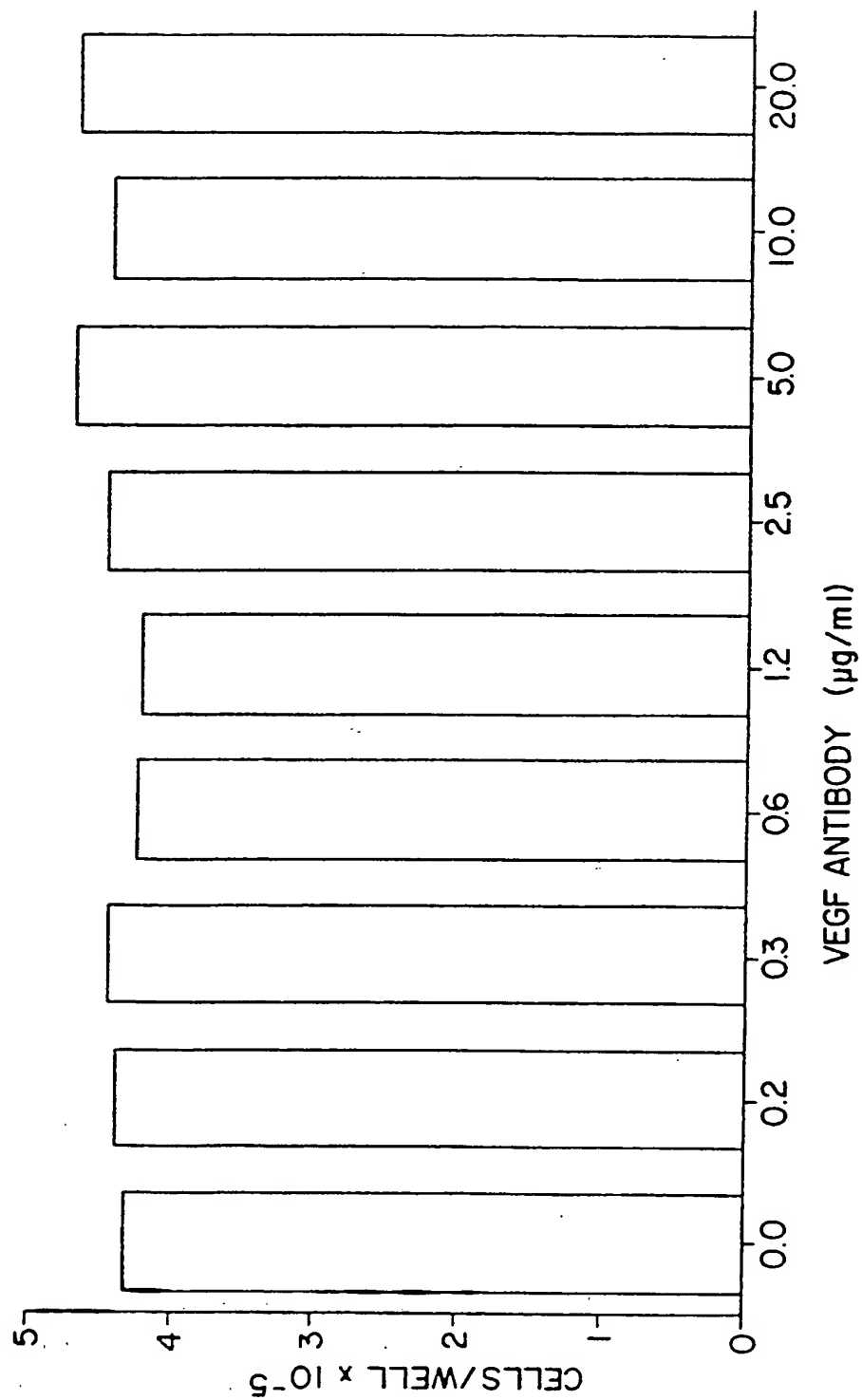


FIG. 8

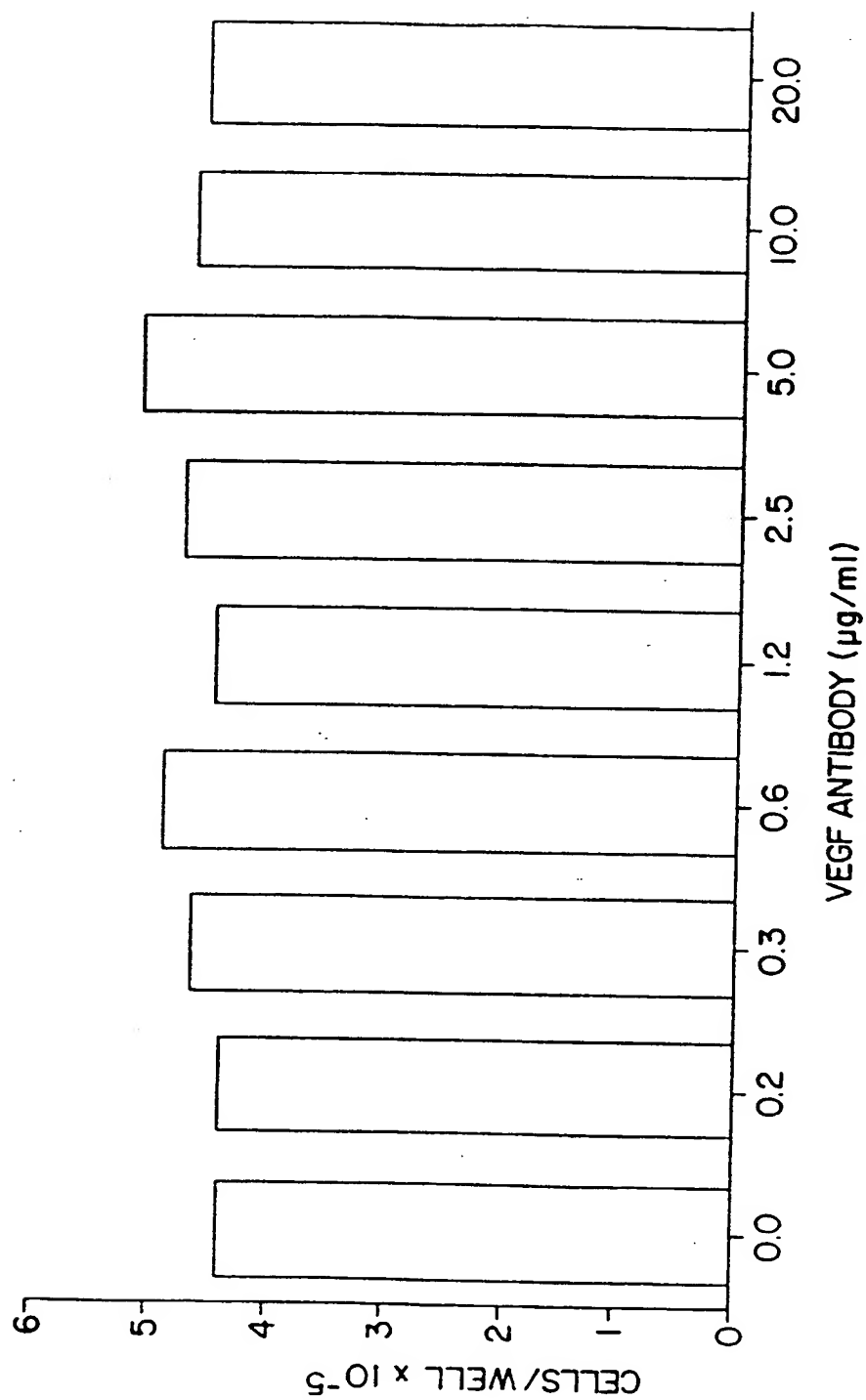


FIG. 9

Sample Type	Sample #	Assay Date	Syn. Fluid	Syn. Fluid + mAB VEGF	% Suppression
Rheumatoid Syn. Fluid	318	5.7.92	5.2 ± 0.2	2.7 ± 0.3	48
	150	5.7.92	7.0 ± 0.3	2.8 ± 0.4	60
	312	5.7.92	6.7 ± 0.4	3.7 ± 0.3	45
	264	5.7.92	6.2 ± 0.4	3.1 ± 0.3	50
	267	5.7.92	5.7 ± 0.6	4.4 ± 0.3	23
	202	5.22.92	10.0 ± 0.5	3.4 ± 0.6	66
	314	5.22.92	7.5 ± 0.3	3.1 ± 0.6	59
	237	5.22.92	6.1 ± 0.5	2.2 ± 0.3	64
	206	5.22.92	6.7 ± 0.5	2.2 ± 0.3	67
	317	5.22.92	5.2 ± 0.3	2.5 ± 0.6	52
Osteoarthritis Syn. Fluid	165	6.2.92	4.0 ± 0.3	2.8 ± 0.4	30
	211	6.2.92	3.4 ± 0.5	3.0 ± 0.2	11.7
	195	6.2.92	3.5 ± 0.2	3.3 ± 0.3	5.7
	122	6.2.92	3.7 ± 0.3	3.2 ± 0.4	13.5
	16	6.2.92	4.1 ± 0.3	3.8 ± 0.5	7.3

Mean % Suppression for RA Fluids 53.4 ± 4.2
Mean % Suppression for OA Fluids 13.6 ± 3.9
Synovial fluids were diluted 1:50.

Controls:

6.2.92	PBS	3.3 ± 0.30
	bFGF 1 µg/ml	3.7 ± 0.38
5.22.92	PBS	1.2 ± 0.38
	bFGF 1 µg/ml	7.8 ± 0.31
5.2.92	PBS	1.3 ± 0.18
	bFGF 1 µg/ml	9.0 ± 0.41

FIG. 10

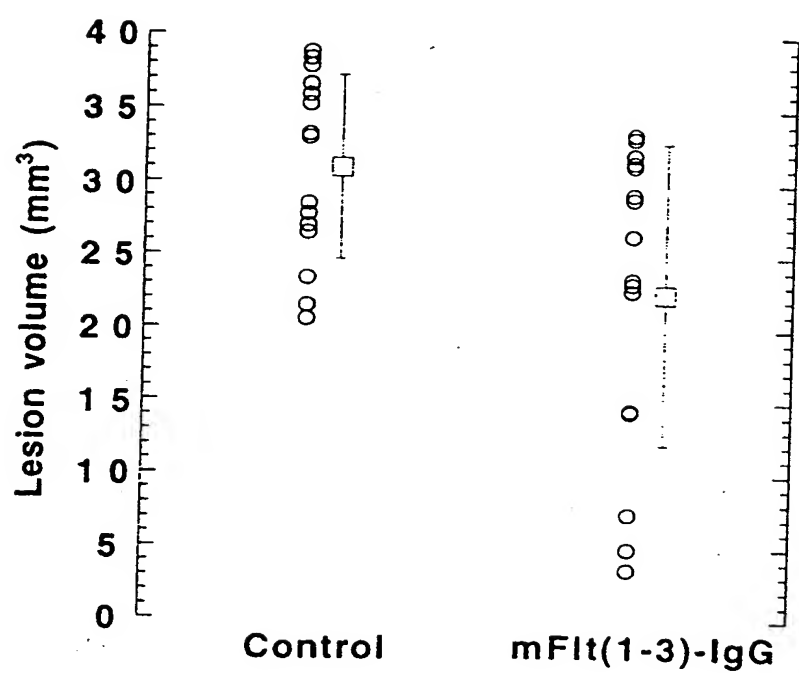


FIG. 11

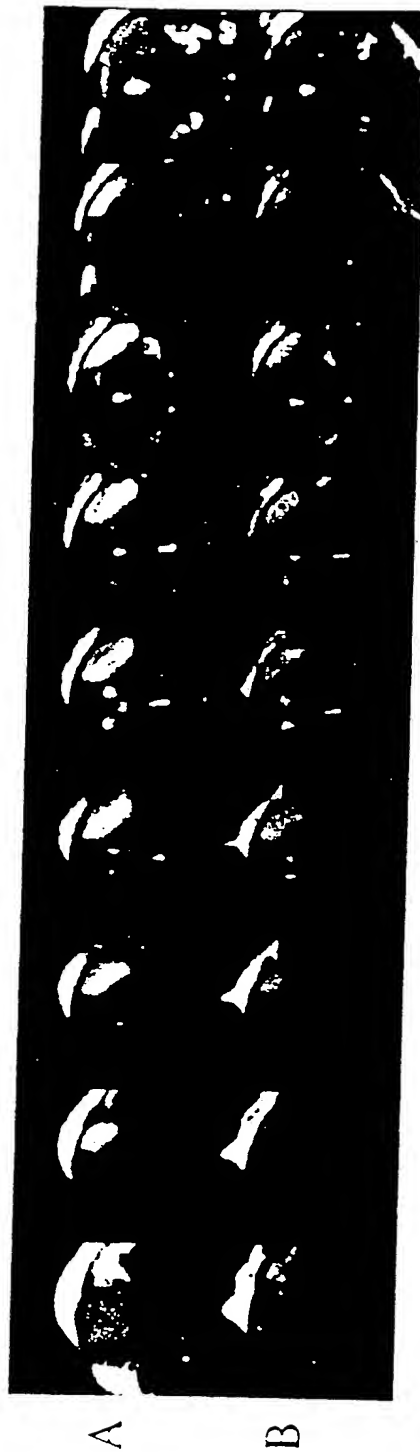


FIG. 12

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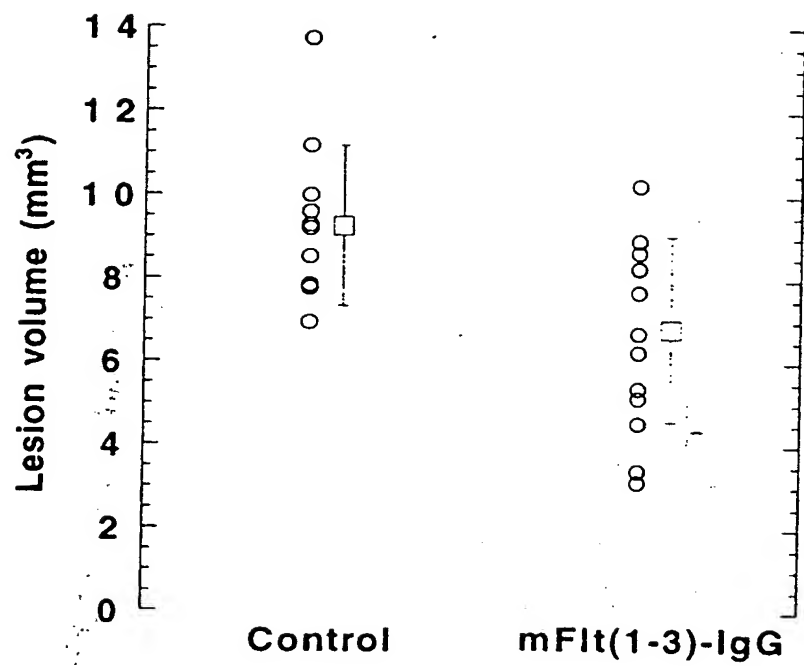


FIG. 13

□ = differences from F)-12

```

      10      20      30
F(ab)-12 DIQMTQSPSSLSASVGDRTITCSASQDISNYLNWYQQ
Y0243-1  DIQMTQSPSSLSASVGDRTITCSASQDISNYLNWYQQ
Y0238-3  DIQMTQSPSSLSASVGDRTITCSASQDISNYLNWYQQ
Y0313-1  DIQMTQSPSSLSASVGDRTITCSASQDISNYLNWYQQ
Y0317    DIQMTQSPSSLSASVGDRTITCSASQDISNYLNWYQQ

```

```

                        CDR-L1
      40      50      60      70
F(ab)-12 KPGKAPKVLIIYFTSSLHSGVPSRFSGSGSGTDFTLTIS
Y0243-1  KPGKAPKVLIIYFTSSLHSGVPSRFSGSGSGTDFTLTIS
Y0238-3  KPGKAPKVLIIYFTSSLHSGVPSRFSGSGSGTDFTLTIS
Y0313-1  KPGKAPKVLIIYFTSSLHSGVPSRFSGSGSGTDFTLTIS
Y0317    KPGKAPKVLIIYFTSSLHSGVPSRFSGSGSGTDFTLTIS

```

FIG. 14A

```

                        CDR-L2
      80      90      100
F(ab)-12 SLQPEDFATYYCQQYSTVPWTFGQGTKVEIKRTV
Y0243-1  SLQPEDFATYYCQQYSTVPWTFGQGTKVEIKRTV
Y0238-3  SLQPEDFATYYCQQYSTVPWTFGQGTKVEIKRTV
Y0313-1  SLQPEDFATYYCQQYSTVPWTFGQGTKVEIKRTV
Y0317    SLQPEDFATYYCQQYSTVPWTFGQGTKVEIKRTV

```

CDR-L3

```

      10      20      30
F(ab)-12 EVQLVESGGGLVQPGGSLRLSCAASGYTFTNYGMNWVR
Y0243-1  EVQLVESGGGLVQPGGSLRLSCAASGYTFTNYGMNWVR
Y0238-3  EVQLVESGGGLVQPGGSLRLSCAASGYTFTNYGMNWVR
Y0313-1  EVQLVESGGGLVQPGGSLRLSCAASGYTFTNYGMNWVR
Y0317    EVQLVESGGGLVQPGGSLRLSCAASGYTFTNYGMNWVR

```

```

                        CDR-H1
      40      50      60      70
F(ab)-12 QAPGKGLEWVGWINTYTGEPTYAADFKRRFTFSLDTSKSTA
Y0243-1  QAPGKGLEWVGWINTYTGEPTYAADFKRRFTFSLDTSKSTA
Y0238-3  QAPGKGLEWVGWINTYTGEPTYAADFKRRFTFSLDTSKSTA
Y0313-1  QAPGKGLEWVGWINTYTGEPTYAADFKRRFTFSLDTSKSTA
Y0317    QAPGKGLEWVGWINTYTGEPTYAADFKRRFTFSLDTSKSTA

```

FIG. 14B

```

      80      90      100      110      CDR-7
F(ab)-12 YLQMNSLRAEDTAVYYCAKYPHYGSSHWYFDVWGQGTL
Y0243-1  YLQMNSLRAEDTAVYYCAKYPHYGSSHWYFDVWGQGTL
Y0238-3  YLQMNSLRAEDTAVYYCAKYPHYGSSHWYFDVWGQGTL
Y0313-1  YLQMNSLRAEDTAVYYCAKYPHYGSSHWYFDVWGQGTL
Y0317    YLQMNSLRAEDTAVYYCAKYPHYGSSHWYFDVWGQGTL

```

CDR-H3

FIG. 15A

■ = differences from F(ab)-12

F(ab)-12 10 20 30
 Y0192 DIQMTQSPSSLSASVGDRVTITCSASQDISNYLNWYQQ
 Y0238-3 DIQ~~ET~~TQSPSSLSASVGDRVTITCRANEQ~~LS~~SNYLNWYQQ
 Y0239-19 DIQ~~ET~~TQSPSSLSASVGDRVTITCRANEQ~~LS~~SNYLNWYQQ
 Y0313-2 DIQ~~ET~~TQSPSSLSASVGDRVTITC~~RANEQLS~~SNYLNWYQQ

CDR-L1

F(ab)-12 40 50 60 70
 Y0192 KPGKAPKVLIIYFTSSLHSGVPSRFSGSGSGTDFTLTIS
 Y0238-3 KPGKAPKVLIIYFTSSLHSGVPSRFSGSGSGTDFTLTIS
 Y0239-19 KPGKAPKVLIIYFTSSLHSGVPSRFSGSGSGTDFTLTIS
 Y0313-2 KPGKAPKVLIIYFTSSLHSGVPSRFSGSGSGTDFTLTIS

CDR-L2

F(ab)-12 80 90 100
 Y0192 SLQPEDFATYYCQQYSTVPWTFGQGTKVEIKRTV
 Y0238-3 SLQPEDFATYYCQQYSTVPWTFGQGTKVEIKRTV
 Y0239-19 SLQPEDFATYYCQQYSTVPWTFGQGTKVEIKRTV
 Y0313-2 SLQPEDFATYYCQQYSTVPWTFGQGTKVEIKRTV

CDR-L3

FIG. 15B

F(ab)-12 10 20 30
 Y0192 EVQLVESGGGLVQPGGSLRLSCAASGYTFTNYGMNWVR
 Y0238-3 EVQLVESGGGLVQPGGSLRLSCAASGYTFTNYGMNWVR
 Y0239-19 EVQLVESGGGLVQPGGSLRLSCAASGYTFTNYGMNWVR
 Y0313-2 EVQLVESGGGLVQPGGSLRLSCAASGYTFTNYGMNWVR

CDR-H1

F(ab)-12 40 50 60 70
 Y0192 QAPGKGLEWVGWINTYTGEPTYAADFKRRFTFSLDTSKSTA
 Y0238-3 QAPGKGLEWVGWINTYTGEPTYAADFKRRFTFSLDTSKSTA
 Y0239-19 QAPGKGLEWVGWINTYTGEPTYAADFKRRFTFSLDTSKSTA
 Y0313-2 QAPGKGLEWVGWINTYTGEPTYAADFKRRFTFSLDTSKSTA

CDR-H2

CDR-7

F(ab)-12 80 90 100
 Y0192 YLQMNSLRAEDTAVYYCAKYPHYGYG---SSHWFYFDVWGQGT
 Y0238-3 YLQMNSLRAEDTAVYYCAKYPHYGYG---SSHWFYFDVWGQGT
 Y0239-19 YLQMNSLRAEDTAVYYCAKYPHYGYG---SSHWFYFDVWGQGT
 Y0313-2 YLQMNSLRAEDTAVYYCAKYPHYGYG---SSHWFYFDVWGQGT

CDR-H3

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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁷ : A61K 39/395, 38/17, A61P 7/10, C07K 19/00 // 14/71	A3	(11) International Publication Number: WO 00/37502 (43) International Publication Date: 29 June 2000 (29.06.00)
(21) International Application Number: PCT/US99/29475 (22) International Filing Date: 9 December 1999 (09.12.99) (30) Priority Data: 09/218,481 22 December 1998 (22.12.98) US (71) Applicant: GENENTECH, INC. [US/US]; 1 DNA Way, South San Francisco, CA 94080-4990 (US). (72) Inventors: VAN BRUGGEN, Nicholas; 2701 Larkin Street, #302, San Francisco, CA 94109 (US). FERRARA, Napoleone; 2090 Pacific Avenue #704, San Francisco, CA 94109 (US). (74) Agents: MARSCHANG, Diane, L. et al.; Genentech, Inc., 1 DNA Way, South San Francisco, CA 94080-4990 (US).		(81) Designated States: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG). Published <i>With international search report.</i> (88) Date of publication of the international search report: 23 November 2000 (23.11.00)
(54) Title: VASCULAR ENDOTHELIAL CELL GROWTH FACTOR ANTAGONISTS AND USES THEREOF (57) Abstract The present invention provides vascular endothelial cell growth factor (VEGF) antagonists and methods of using VEGF antagonists. VEGF antagonists contemplated by the invention include VEGF antibodies and VEGF receptor fusion proteins. Methods of treating edema and stroke using VEGF antagonists are also provided.		

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INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 99/29475

A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 A61K39/395 A61K38/17 A61P7/10 C07K19/00 //C07K14/71

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

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IPC 7 C07K A61K

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Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 94 10202 A (GENENTECH INC) 11 May 1994 (1994-05-11) page 2, line 38 - page 3, line 4 page 5, line 8 - line 16 page 6, line 8 - line 35 page 8, line 24 - line 27 page 16, line 25 - line 37 page 17, line 15 - line 28 ---	1-29
X	WO 98 16551 A (FERRARA NAPOLEONE ; NGUYEN FRANCIS HUNG (US); GENENTECH INC (US); K) 23 April 1998 (1998-04-23) abstract; claims 1-15 page 5, line 15 - line 31 ---	1-4, 22
	--- -/-	

☒ Further documents are listed in the continuation of box C.

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Date of the actual completion of the international search

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Date of mailing of the international search report

23. 06. 00

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INTERNATIONAL SEARCH REPORT

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PCT/US 99/29475

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 98 45331 A (CHEN YVONNE MAN YEE ;BACA MANUEL (US); GENENTECH INC (US); WELLS J) 15 October 1998 (1998-10-15) the whole document ----	1-29
A	WO 97 44453 A (GENENTECH INC ;DAVIS SMYTH TERRI LYNN (US); CHEN HELEN HSIFEI (US)) 27 November 1997 (1997-11-27) page 39; claims 1,10 ----	1-29
A	BERKMAN, RICHARD A. ET AL: "Expression of the vascular permeability factor/vascular endothelial growth factor gene in central nervous system neoplasms." JOURNAL OF CLINICAL INVESTIGATION, (1993) VOL. 91, NO. 1, PP. 153-159. , XP000909359 the whole document ----	1-29
P,X	VAN BRUGGEN N ET AL: "VEGF antagonism reduces edema formation and tissue damage after ischemia /reperfusion injury in the mouse brain." JOURNAL OF CLINICAL INVESTIGATION, (1999 DEC) 104 (11) 1613-20. , XP000909358 abstract page 1619, right-hand column -----	1-29

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 99/29475

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

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Although claims 1-29 are directed to a method of treatment of the mammal body, the search has been carried out and based on the alleged effects of the compound.
2. ☐ Claims Nos.:
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3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

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- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/US 99/29475

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9410202 A	11-05-1994	CA 2145985 A	11-05-1994
		CZ 9501057 A	13-12-1995
		HU 70810 A	28-11-1995
		AU 687727 B	05-03-1998
		AU 2928992 A	24-05-1994
		BG 99605 A	29-02-1996
		BR 9207175 A	12-12-1995
		EP 0666868 A	16-08-1995
		FI 951987 A	26-04-1995
		JP 8502514 T	19-03-1996
		NO 951609 A	27-04-1995
		SK 55195 A	09-08-1995
WO 9816551 A	23-04-1998	AU 718327 B	13-04-2000
		AU 5152398 A	11-05-1998
		EP 0931092 A	28-07-1999
WO 9845331 A	15-10-1998	AU 7100798 A	30-10-1998
		AU 7102398 A	30-10-1998
		EP 0973804 A	26-01-2000
		EP 0971959 A	19-01-2000
		NO 994869 A	06-12-1999
		NO 994870 A	06-12-1999
		WO 9845332 A	15-10-1998
		ZA 9802907 A	09-10-1998
WO 9744453 A	27-11-1997	ZA 9802908 A	21-01-1999
		AU 717112 B	16-03-2000
		AU 3060497 A	09-12-1997
		EP 0907733 A	14-04-1999
		JP 2000502357 T	29-02-2000
		NZ 332779 A	29-06-1999
		US 5952199 A	14-09-1999

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

CORRECTED VERSION

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(84) Designated States (*regional*): ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published:
— with international search report

(71) Applicant: GENENTECH, INC. [US/US]; 1 DNA Way, South San Francisco, CA 94080-4990 (US).

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(72) Inventors: VAN BRUGGEN, Nicholas; 2701 Larkin Street, #302, San Francisco, CA 94109 (US). FERRARA, Napoleone; 2090 Pacific Avenue #704, San Francisco, CA 94109 (US).

(48) Date of publication of this corrected version:
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(74) Agents: MARSCHANG, Diane, L. et al.; Genentech, Inc., 1 DNA Way, South San Francisco, CA 94080-4990 (US).

(15) Information about Correction:
see PCT Gazette No. 35/2002 of 29 August 2002, Section II

(81) Designated States (*national*): AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK,

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) Title: VASCULAR ENDOTHELIAL CELL GROWTH FACTOR ANTAGONISTS AND USES THEREOF

(57) Abstract: The present invention provides vascular endothelial cell growth factor (VEGF) antagonists and methods of using VEGF antagonists. VEGF antagonists contemplated by the invention include VEGF antibodies and VEGF receptor fusion proteins. Methods of treating edema and stroke using VEGF antagonists are also provided.

VASCULAR ENDOTHELIAL CELL GROWTH FACTOR ANTAGONISTS AND USES THEREOF

Field of the Invention

5 The present invention relates to vascular endothelial cell growth factor (VEGF) antagonists, to therapeutic compositions comprising the antagonists, and to methods of use of the antagonists for diagnostic and therapeutic purposes. In particular, the present invention relates to methods of treatment of stroke or edema using VEGF antagonists.

10

Background of the Invention

The two major cellular components of the vasculature are the endothelial and smooth muscle cells. The endothelial cells form the lining of the inner surface of all blood vessels, and constitute a nonthrombogenic interface between blood and tissue. In addition, 15 endothelial cells are an important component for the development of new capillaries and blood vessels. Thus, endothelial cells proliferate during the angiogenesis, or neovascularization, associated with tumor growth and metastasis, as well as a variety of non-neoplastic diseases or disorders.

20

Various naturally occurring polypeptides reportedly induce the proliferation of endothelial cells. Among those polypeptides are the basic and acidic fibroblast growth factors (FGF), Burgess and Maciag, Annual Rev. Biochem., 58:575 (1989), platelet-derived endothelial cell growth factor (PD-ECGF), Ishikawa, et al., Nature, 338:557 (1989), and 25 vascular endothelial growth factor (VEGF), Leung, et al., Science 246:1306 (1989); Ferrara & Henzel, Biochem. Biophys. Res. Commun. 161:851 (1989); Tischer, et al., Biochem. Biophys. Res. Commun. 165:1198 (1989); Ferrara, et al., PCT Pat. Pub. No. WO 90/13649 (published November 15, 1990).

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VEGF was first identified in media conditioned by bovine pituitary follicular or folliculostellate cells. Biochemical analyses indicate that bovine VEGF is a dimeric protein with an apparent molecular mass of approximately 45,000 Daltons, and with an apparent mitogenic specificity for vascular endothelial cells. DNA encoding bovine VEGF was isolated by 35 screening a cDNA library prepared from such cells, using oligonucleotides based on the amino-terminal amino acid sequence of the protein as hybridization probes.

Human VEGF was obtained by first screening a cDNA library prepared from human cells, using bovine VEGF cDNA as a hybridization probe. One cDNA identified thereby encodes a 165-amino acid protein having greater than 95% homology to bovine VEGF, which protein is referred to as human VEGF (hVEGF). The mitogenic activity of human VEGF was confirmed by expressing the human VEGF cDNA in mammalian host cells. Media conditioned by cells transfected with the human VEGF cDNA promoted the proliferation of capillary endothelial cells, whereas control cells did not. See, Leung, *et al.*, Science 246:1306 (1989).

Several additional cDNAs were identified in human cDNA libraries that encode 121-, 189-, and 206-amino acid isoforms of hVEGF (also collectively referred to as hVEGF-related proteins). The 121-amino acid protein differs from hVEGF by virtue of the deletion of the 44 amino acids between residues 116 and 159 in hVEGF. The 189-amino acid protein differs from hVEGF by virtue of the insertion of 24 amino acids at residue 116 in hVEGF, and apparently is identical to human vascular permeability factor (hVPF). The 206-amino acid protein differs from hVEGF by virtue of an insertion of 41 amino acids at residue 116 in hVEGF. Houck, *et al.*, Mol. Endocrin. 5:1806 (1991); Ferrara, *et al.*, J. Cell. Biochem. 47:211 (1991); Ferrara, *et al.*, Endocrine Reviews 13:18 (1992); Keck, *et al.*, Science 246:1309 (1989); Connolly, *et al.*, J. Biol. Chem. 264:20017 (1989); Keck, *et al.*, EPO Pat. Pub. No. 0 370 989 (published May 30, 1990).

Receptors for VEGF have been described in the literature. Two such receptors, flt-1 and flk-1, have been found to mediate VEGF effects [DeVries *et al.*, Science 255:989 (1992); Shibuya *et al.*, Oncogene 5:519 (1990); Matthews *et al.*, Proc. Natl. Acad. Sci. 88:9026 (1991); Terman *et al.*, Oncogene 6:1677 (1991); Terman *et al.*, Biochem. Biophys. Res. Comm. 187:1579 (1992); Neufeld *et al.*, Prog. Growth Factor Res. 5:89-97 (1994); Waltenberger *et al.*, J. Biol. Chem. 269:26988 (1994); Quinn *et al.*, Proc. Natl. Acad. Sci. 90:7533 (1993)], but their regulation and mechanisms are not yet fully understood. Lennmyr *et al.*, J. Neuropathology and Exp. Neurology 57:874-882 (1998). Both the flt-1 and flk-1 receptors are membrane-spanning receptors and belong to the class III tyrosine kinase receptor family. Barleon *et al.*, J. Cell Biochem. 54:56 (1994); Neufeld *et al.*, *supra*.

VEGF not only stimulates vascular endothelial cell proliferation, but also induces angiogenesis. Angiogenesis, which involves the

formation of new blood vessels from preexisting endothelium, is an important component of a variety of diseases and disorders including tumor growth and metastasis, rheumatoid arthritis, psoriasis, atherosclerosis, diabetic retinopathy, retrolental fibroplasia, neovascular glaucoma, age-related macular degeneration, hemangiomas, immune rejection of transplanted corneal tissue and other tissues, and chronic inflammation.

In the case of tumor growth, angiogenesis appears to be crucial for the transition from hyperplasia to neoplasia, and for providing nourishment to the growing solid tumor. Folkman, *et al.*, *Nature* 339:58 (1989). Angiogenesis also allows tumors to be in contact with the vascular bed of the host which may provide a route for metastasis of the tumor cells. Evidence for the role of angiogenesis in tumor metastasis is provided, for example, by studies showing a correlation between the number and density of microvessels in histologic sections of invasive human breast carcinoma and actual presence of distant metastases. Weidner, *et al.*, *New Engl. J. Med.* 324:1 (1991).

VEGF has also been reported to be involved in endothelial and vascular permeability. See, Ferrara *et al.*, *Endocrine Reviews* 18:4-25 (1997); Dobrogowska *et al.*, *J. Neurocytology* 27:163 (1998). Although not fully understood, VEGF is believed to increase endothelial cell leakage in skin, retina, and tumor tissues. Collins *et al.*, *Brit. J. Pharmacology* 109:195 (1993); Connolly *et al.*, *J. Clin. Invest.* 84:1470 (1989); Shweiki *et al.*, *Nature* 359:843 (1992); Monacci *et al.*, *Am. J. Physiol.* 264:C995 (1993); Stone *et al.*, *J. Neurosci.* 15:4738 (1995); Detmar *et al.*, *J. Invest. Dermatol.* 108:263 (1997); Weindel *et al.*, *Neurosurgery* 35:437 (1994). The potential effects and role of VEGF (and its receptors, particularly, the *flt-1* receptor), on endothelial cell and blood-brain barrier permeability have also been examined. See, e.g., Rosenstein *et al.*, *Proc. Natl. Acad. Sci.* 95:7086 (1998); Dobrogowska, *supra*; Kovacs *et al.*, *Stroke* 27:1865 (1996). Relatively diffuse VEGF mRNA expression has been observed in adult rat brain but at somewhat low abundance. Monacci *et al.*, *Am. J. Physiol.* 146:368-378 (1993). However, reduced oxygen tension has been shown to trigger VEGF expression [Dor and Keshet, *Trends in Cardiovascular Med.*, 7:289-294 (1998)] and enhanced levels of VEGF, *flt-1*, and *flk-1* have been shown to occur in the rat brain following the induction of focal cerebral ischemia. Hayashi *et al.*, *Stroke* 28:2039 (1997); Kovacs *et al.*, *supra*; Lennmyr *et al.*, *J.*

Neuropathology and Experimental Neurology, 57:874 (1998). The role of VEGF in the pathogenesis of stroke and BBB breakdown has been unclear with contradictory experimental observations cited in the literature. For example, Nag et al., J. Neuropathology and Experimental Neurology 56:912 (1997), in their cortical cold-injury rat model, demonstrated the presence of mural VEGF in permeable pial vessels and arterioles within the damaged tissue and, from this observation, it was inferred that VEGF is one of several factors that may mediate BBB breakdown and edema formation. On the other hand, in Hayashi et al., J. Cerebral Blood Flow and Metabolism, 18:887 (1998), it is reported that VEGF itself, when applied topically on the surface of a reperfused rat brain after transient cerebral artery occlusion, reduced ischemic brain damage, infarct volume and edema formation.

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Summary of the Invention

The present invention provides antagonists of VEGF, including (a) antibodies and variants thereof which are capable of specifically binding to hVEGF, hVEGF receptor, or a complex comprising hVEGF in association with hVEGF receptor, (b) hVEGF receptor and variants thereof, and (c) hVEGF variants. The antagonists inhibit, sequester or neutralize the mitogenic, angiogenic, vascular permeability or other biological activity of hVEGF, and thus are useful for the treatment of diseases or conditions characterized by undesirable excessive neovascularization, including by way of example, tumors, and especially solid malignant tumors, rheumatoid arthritis, psoriasis, atherosclerosis, diabetic and other retinopathies, retrolental fibroplasia, age-related macular degeneration, neovascular glaucoma, hemangiomas, thyroid hyperplasias (including Grave's disease), corneal and other tissue transplantation, and chronic inflammation. The antagonists also are useful for the treatment of diseases or conditions such as edema which may be associated with, e.g., tumors, stroke, head trauma, ascites associated with malignancies, Meigs' syndrome, lung inflammation, nephrotic syndrome, pericardial effusion (such as that associated with pericarditis), and pleural effusion.

In other aspects, the VEGF antagonists are polyspecific monoclonal antibodies which are capable of binding to (a) a non-hVEGF epitope, for example, an epitope of a protein involved in thrombogenesis or thrombolysis, or a tumor cell surface antigen, or to (b) hVEGF, hVEGF

receptor, or a complex comprising hVEGF in association with hVEGF receptor.

In still other aspects, the VEGF antagonists are conjugated with a cytotoxic moiety.

5 In another aspect, the invention concerns isolated nucleic acids encoding the monoclonal antibodies as hereinbefore described, and hybridoma cell lines which produce such monoclonal antibodies.

10 In another aspect, the invention concerns compositions, such as pharmaceutical compositions, comprising a VEGF antagonist in an amount effective in reducing or eliminating hVEGF-mediated mitogenic, angiogenic, or other biological activity in a mammal.

15 In a different aspect, the invention concerns methods of treatment comprising administering to a mammal, preferably a human patient in need of such treatment, an effective amount of a VEGF antagonist. If desired, the VEGF antagonist is co-administered, either simultaneously or sequentially, with one or more other VEGF antagonists, anti-tumor or anti-angiogenic substances, or therapies suitable for the disease or condition being treated.

20 In another aspect, the invention concerns a method for detecting hVEGF in a test sample by means of contacting the test sample with an antibody capable of binding specifically to hVEGF and determining the extent of such binding.

Brief Description of the Drawings

25 Figure 1 shows the effect of anti-hVEGF monoclonal antibodies (A4.6.1 or B2.6.2) or an irrelevant anti-hepatocyte growth factor antibody (anti-HGF) on the binding of the anti-hVEGF monoclonal antibodies to hVEGF.

30 Figure 2 shows the effect of anti-hVEGF monoclonal antibodies (A4.6.1 or B2.6.2) or an irrelevant anti-HGF antibody on the biological activity of hVEGF in cultures of bovine adrenal cortex capillary endothelial (ACE) cells.

Figure 3 shows the effect of anti-hVEGF monoclonal antibodies (A4.6.1, B2.6.2, or A2.6.1) on the binding of hVEGF to bovine ACE cells.

35 Figure 4 shows the effect of A4.6.1 anti-hVEGF monoclonal antibody treatment on the rate of growth of growth of NEG55 tumors in mice.

Figure 5 shows the effect of A4.6.1 anti-hVEGF monoclonal antibody treatment on the size of NEG55 tumors in mice after five weeks of treatment.

Figure 6 shows the effect of A4.6.1 anti-hVEGF monoclonal antibody (VEGF Ab) treatment on the growth of SK-LMS-1 tumors in mice.

Figure 7 shows the effect of varying doses of A4.6.1 anti-hVEGF monoclonal antibody (VEGF Ab) treatment on the growth of A673 tumors in mice.

Figure 8 shows the effect of A4.6.1 anti- hVEGF monoclonal antibody on the growth and survival of NEG55 (G55) glioblastoma cells in culture.

Figure 9 shows the effect of A4.6.1 anti- hVEGF monoclonal antibody on the growth and survival of A673 rhabdomyosarcoma cells in culture.

Figure 10 shows the effect of A4.6.1 anti-hVEGF monoclonal antibody on human synovial fluid-induced chemotaxis of human endothelial cells.

Figure 11 shows the effect of flt-IgG treatment on the extent of edematous tissue as depicted by high signal intensity on the T2-weighted MR image.

Figure 12 shows representative T2-weighted MR images recorded 24 hours following onset of ischemia for both the control (top panel) and treatment group (bottom panel), showing reduction in edematous tissue in the treatment group.

Figure 13 shows the effect of flt-IgG treatment on the size of infarction determined using high resolution anatomical MRI 8-12 weeks following onset of ischemia.

Figures 14A-B show an alignment of the amino acid sequences for the light and heavy variable domains respectively of affinity matured anti-VEGF antibodies compared to the F(ab)-12 antibody (SEQ ID NO:1 shown in Figure 14A; SEQ ID NO:9 shown in Figure 14B). CDRs are underlined and designated by L, light, or H, heavy chains, and numbers 1-3. The affinity matured sequences are designated YO243-1 (SEQ ID NO :2 shown in Figure 14A; SEQ ID NO:10 shown in Figure 14B); YO238-3 (SEQ ID NO:3 shown in Figure 14A; SEQ ID NO:11 shown in Figure 14B); YO313-1 (SEQ ID NO:4 shown in Figure 14A; SEQ ID NO:12 shown in Figure 14B); and YO317 (SEQ ID NO:5 shown in Figure 14A; SEQ ID NO:13 shown in Figure 14B). Differences from F(ab)-12 are shown in shaded boxes.

Figures 15A-B show an alignment of the amino acid sequences for the light and heavy variable domains respectively of affinity matured anti-VEGF antibodies compared to the F(ab)-12 antibody (SEQ ID NO:1 shown in

Figures 14A and 15A; SEQ ID NO:9 shown in Figures 14B and 15B). CDRs are underlined and designated by L, light, or H, heavy chains, and numbers 1-3. The affinity matured sequences are designated YO192 (SEQ ID NO:6 shown in Figure 15A; SEQ ID NO:14 shown in Figure 15B); YO238-3 (SEQ ID NO:3 shown in Figures 14A and 14B; SEQ ID NO:11 shown in Figures 14B and 15B); YO239-19 (SEQ ID NO:7 shown in Figure 15A; SEQ ID NO:15 shown in Figure 15B); and YO313-2 (SEQ ID NO:8 shown in Figure 15A; SEQ ID NO:16 shown in Figure 15B). Differences from F(ab)-12 are shown in shaded boxes.

Detailed Description of the Invention

The present invention provides antagonists of hVEGF which are capable of inhibiting, sequestering, or neutralizing one or more of the biological activities of hVEGF. Antagonists of hVEGF act by interfering with the binding of hVEGF to a cellular receptor, by incapacitating or killing cells which have been activated by hVEGF, or by interfering with vascular endothelial cell activation after hVEGF binding to a cellular receptor. All such points of intervention by an hVEGF antagonist shall be considered equivalent for purposes of this invention. Thus, included within the scope of the invention are antibodies, monoclonal antibodies and humanized antibodies, or fragments thereof, that bind to hVEGF, hVEGF receptor, or a complex comprising hVEGF in association with hVEGF receptor. Also included within the scope of the invention are fragments and amino acid sequence variants of hVEGF that bind to hVEGF receptor but which do not exhibit a biological activity of native hVEGF. Also included within the scope of the invention are hVEGF receptor and fragments and amino acid sequence variants thereof which are capable of binding hVEGF.

The term "hVEGF" as used herein refers to the 165-amino acid human vascular endothelial cell growth factor, and related 121-, 189-, and 206-amino acid vascular endothelial cell growth factors, as described by Leung, et al., Science 246:1306 (1989), and Houck, et al., Mol. Endocrin. 5:1806 (1991), together with the naturally occurring allelic and processed forms of those growth factors.

The term "hVEGF receptor" or "hVEGFr" as used herein refers to a cellular receptor for hVEGF, ordinarily a cell-surface receptor found on vascular endothelial cells, as well as fragments and variants thereof which retain the ability to bind hVEGF. Typically, the hVEGF receptors and fragments and variants thereof that are hVEGF antagonists will be in isolated form, rather than being integrated into a cell membrane or fixed

to a cell surface as may be the case in nature. One example of a hVEGF receptor is the fms-like tyrosine kinase (flt or flt-1), a transmembrane receptor in the tyrosine kinase family. DeVries, et al., Science 255:989 (1992); Shibuya, et al., Oncogene 5:519 (1990). The full length flt receptor comprises an extracellular domain, a transmembrane domain, and an intracellular domain with tyrosine kinase activity. The extracellular domain is involved in the binding of hVEGF, whereas the intracellular domain is involved in signal transduction.

Another example of a hVEGF receptor is the flk-1 receptor (also referred to as KDR). Matthews, et al., Proc. Nat. Acad. Sci. 88:9026 (1991); Terman, et al., Oncogene 6:1677 (1991); Terman, et al., Biochem. Biophys. Res. Commun. 187:1579 (1992).

Binding of hVEGF to the flt receptor results in the formation of at least two high molecular weight complexes, having apparent molecular weight of 205,000 and 300,000 Daltons. The 300,000 Dalton complex is believed to be a dimer comprising two receptor molecules bound to a single molecule of hVEGF.

Variants of hVEGFr also are included within the scope hereof. Representative examples include truncated forms of a receptor in which at least the transmembrane and cytoplasmic domains are deleted from the full length receptor molecule, and fusions proteins in which non- hVEGFr polymers or polypeptides are conjugated to the hVEGFr or, preferably, truncated forms thereof. An example of such a non- hVEGF polypeptide is an immunoglobulin. In that case, for example, an extracellular domain sequence of the hVEGFr is substituted for the Fv domain of an immunoglobulin light or (preferably) heavy chain, with the C-terminus of the receptor extracellular domain covalently joined to the amino terminus of the CH1, hinge, CH2 or other fragment of the heavy chain. Such variants are made in the same fashion as known immunoadhesins. See e.g., Gascoigne, et al., Proc. Nat. Acad. Sci. 84:2936 (1987); Capon, et al., Nature 337:525 (1989); Aruffo, et al., Cell 61:1303 (1990); Ashkenazi, et al., Proc. Nat. Acad. Sci. 88:10535 (1991); Bennett, et al., J. Biol. Chem. 266:23060 (1991). Examples of various flt-IgG fusion proteins are described in Example 3 below. Truncated forms of the extracellular domain of the hVEGF receptor contemplated for use in the invention include ECD fragments (for instance, having one or more amino acids in the ECD sequence deleted) and ECD forms having one or more immunoglobulin-like domains in the ECD deleted. Example 3B describes,

for instance, a truncated ECD form which includes only the first three immunoglobulin-like domains of flt fused to a Fc-IgG. Preferably, a truncated form of the ECD used in making an antagonist molecule will include sufficient immunoglobulin-like domain(s) to ensure a desired binding to hVEGF.

In other embodiments, the hVEGFr or fragments or variants thereof are conjugated to a non-proteinaceous polymer such as polyethylene glycol (PEG) (see e.g., Davis, et al., U.S. Patent No. 4,179,337; Goodson, et al., BioTechnology 8:343-346 (1990); Abuchowski, et al., J. Biol. Chem. 252:3578 (1977); Abuchowski, et al., J. Biol. Chem. 252:3582 (1977)) or carbohydrates (see e.g., Marshall, et al., Arch. Biochem. Biophys., 167:77 (1975)). This can serve to extend the biological half-life of the hVEGFr and reduce the possibility that the receptor will be immunogenic in the mammal to which it is administered.

The hVEGFr is used in substantially the same fashion as antibodies to hVEGF, taking into account the affinity of the antagonist and its valency for hVEGF. An extracellular domain sequence of hVEGF receptor, either by itself or fused to an immunoglobulin polypeptide or other carrier polypeptide, is especially useful as an antagonist of hVEGF, by virtue of its ability to sequester hVEGF that is present in a host but that is not bound to hVEGFr on a cell surface.

HVEGFr and fragments and variants thereof also are useful in screening assays to identify agonists and antagonists of hVEGF. For example, host cells transfected with DNA encoding hVEGFr (for example, flt or flk-1) overexpress the receptor polypeptide on the cell surface, making such recombinant host cells ideally suited for analyzing the ability of a test compound (for example, a small molecule, linear or cyclic peptide, or polypeptide) to bind to hVEGFr. hVEGFr and hVEGFr fusion proteins, such as an hVEGFr-IgG fusion protein, may be used in a similar fashion. For example, the fusion protein is bound to an immobilized support and the ability of a test compound to displace radiolabeled hVEGF from the hVEGFr domain of the fusion protein is determined.

The term "recombinant" used in reference to hVEGF, hVEGF receptor, antibodies, or other proteins, refers to proteins that are produced by recombinant DNA expression in a host cell. The host cell may be prokaryotic (for example, a bacterial cell such as E. coli) or eukaryotic (for example, a yeast or a mammalian cell).

Antagonist Antibodies

The term "monoclonal antibody" as used herein refers to an antibody obtained from a population of substantially homogeneous antibodies, i.e., the individual antibodies comprising the population are identical in specificity and affinity except for possible naturally occurring mutations that may be present in minor amounts. It should be appreciated that as a result of such naturally occurring mutations and the like, a monoclonal antibody composition of the invention, which will predominantly contain antibodies capable of specifically binding hVEGF, hVEGFr, or a complex comprising hVEGF in association with hVEGFr ("hVEGF-hVEGFr complex"), may also contain minor amounts of other antibodies.

Thus, the modifier "monoclonal" indicates the character of the antibody as being obtained from such a substantially homogeneous population of antibodies, and is not to be construed as requiring production of the antibody by any particular method. For example, monoclonal antibodies of the invention may be made using the hybridoma method first described by Kohler & Milstein, Nature 256:495 (1975), or may be made by recombinant DNA methods. See, e.g., Cabilly, et al., U.S. Pat. No. 4,816,567.

In the hybridoma method, a mouse or other appropriate host animal is immunized with antigen by subcutaneous, intraperitoneal, or intramuscular routes to elicit lymphocytes that produce or are capable of producing antibodies that will specifically bind to the protein(s) used for immunization. Alternatively, lymphocytes may be immunized in vitro. Lymphocytes then are fused with myeloma cells using a suitable fusing agent, such as polyethylene glycol, to form a hybridoma cell. Goding, Monoclonal Antibodies: Principles and Practice, pp.59-103 (Academic Press, 1986).

The antigen may be hVEGF, hVEGFr, or hVEGF-hVEGFr complex. The antigen optionally is a fragment or portion or variant of any one of hVEGF or hVEGFr having one or more amino acid residues that participate in the binding of hVEGF to one of its receptors. For example, immunization with an extracellular domain sequence of an hVEGFr (such as, a truncated hVEGFr polypeptide lacking at least transmembrane and intracellular domains) will be especially useful in producing antibodies that are antagonists of hVEGF, since it is region(s) within the extracellular domain that are involved in hVEGF binding..

Monoclonal antibodies capable of binding hVEGF-hVEGFr complex are useful, particularly if they do not also bind to non-associated (non-complexed) hVEGF and hVEGFr. Such antibodies thus only bind to cells undergoing immediate activation by hVEGF and accordingly are not sequestered by free hVEGF or hVEGFr as is normally found in a mammal. Such antibodies typically bind an epitope that spans one or more points of contact between the receptor and hVEGF. Such antibodies have been produced for other ligand receptor complexes and may be produced here in the same fashion. These antibodies need not, and may not, neutralize or inhibit a biological activity of non-associated hVEGF or hVEGFr, whether or not the antibodies are capable of binding to non-associated hVEGF or hVEGFr.

The hybridoma cells thus prepared are seeded and grown in a suitable culture medium that preferably contains one or more substances that inhibit the growth or survival of the unfused, parental myeloma cells. For example, if the parental myeloma cells lack the enzyme hypoxanthine guanine phosphoribosyl transferase (HGPRT or HPRT), the culture medium for the hybridomas typically will include hypoxanthine, aminopterin, and thymidine (HAT medium), which substances prevent the growth of HGPRT-deficient cells.

Preferred myeloma cells are those that fuse efficiently, support stable high level expression of antibody by the selected antibody-producing cells, and are sensitive to a medium such as HAT medium. Among these, preferred myeloma cell lines are murine myeloma lines, such as those derived from MOPC-21 and MPC-11 mouse tumors available from the Salk Institute Cell Distribution Center, San Diego, California USA, SP-2 cells available from the American Type Culture Collection, Manassas, Virginia USA, and P3X63Ag8U.1 cells described by Yelton, *et al.*, Curr. Top. Microbiol. Immunol. 81:1 (1978). Human myeloma and mouse-human heteromyeloma cell lines also have been described for the production of human monoclonal antibodies. Kozbor, J. Immunol. 133:3001 (1984); Brodeur, *et al.*, Monoclonal Antibody Production Techniques and Applications, pp.51-63 (Marcel Dekker, Inc., New York, 1987).

Culture medium in which hybridoma cells are growing is assayed for production of monoclonal antibodies directed against the antigen. Preferably, the binding specificity of monoclonal antibodies produced by hybridoma cells is determined by immunoprecipitation or by an *in vitro* binding assay, such as radioimmunoassay (RIA) or enzyme-linked

immunoabsorbent assay (ELISA). The monoclonal antibodies of the invention are those that preferentially immunoprecipitate hVEGF, hVEGFr, or hVEGF-hVEGFr complex, or that preferentially bind to at least one of those antigens in a binding assay, and that are capable of inhibiting a biological activity of hVEGF.

After hybridoma cells are identified that produce antagonist antibodies of the desired specificity, affinity, and activity, the clones may be subcloned by limiting dilution procedures and grown by standard methods. Goding, Monoclonal Antibodies: Principles and Practice, pp.59-104 (Academic Press, 1986). Suitable culture media for this purpose include, for example, Dulbecco's Modified Eagle's Medium or RPMI-1640 medium. In addition, the hybridoma cells may be grown in vivo as ascites tumors in an animal.

The monoclonal antibodies secreted by the subclones are suitably separated from the culture medium, ascites fluid, or serum by conventional immunoglobulin purification procedures such as, for example, protein A-Sepharose, hydroxylapatite chromatography, gel electrophoresis, dialysis, or affinity chromatography.

DNA encoding monoclonal antibodies of the invention is readily isolated and sequenced using conventional procedures (e.g., by using oligonucleotide probes that are capable of binding specifically to genes encoding the heavy and light chains of murine antibodies). The hybridoma cells of the invention serve as a preferred source of such DNA. Once isolated, the DNA may be placed into expression vectors, which are then transfected into host cells such as simian COS cells, Chinese Hamster ovary (CHO) cells, or myeloma cells that do not otherwise produce immunoglobulin protein, to obtain the synthesis of monoclonal antibodies in the recombinant host cells.

The DNA optionally may be modified in order to change the character of the immunoglobulin produced by its expression. For example, humanized forms of murine antibodies are produced by substituting a complementarity determining region (CDR) of the murine antibody variable domain for the corresponding region of a human antibody. In some embodiments, selected framework region (FR) amino acid residues of the murine antibody also are substituted for the corresponding amino acid residues in the human antibody. Carter, et al., Proc. Nat. Acad. Sci. 89:4285 (1992); Carter, et al., BioTechnology 10:163 (1992). Chimeric forms of murine antibodies also are produced by substituting the coding sequence for selected human

heavy and light constant chain domains in place of the homologous murine sequences. Cabilly, et al., U.S. Pat. No. 4,816,567; Morrison, et al., Proc. Nat. Acad. Sci. 81:6851 (1984).

Particular humanized antibodies contemplated for use in the present invention include the humanized and affinity matured anti- hVEGF antibodies described in published PCT applications WO 98/45331 (published October 15, 1998) and WO 98/45332 (published October 15, 1998). Such humanized or affinity matured anti-VEGF antibodies may be prepared or made using the methods and techniques described in WO 98/45331 and WO 98/45332. Preferably, the anti- hVEGF antibody comprises the humanized F(ab), designated as F(ab)-12, or the affinity matured antibody, designated as YO317, in the above referenced PCT applications. Figures 14A-B and 15A-B illustrate the amino acid sequences (light and heavy chains) for these anti-VEGF antibodies, along with other affinity matured anti-VEGF antibodies, designated as YO192; YO238-3; YO239-19; YO313-2; YO243-1; and YO313-1. All such anti-VEGF antibodies are contemplated for use in the methods described herein. As disclosed in these published PCT applications, several of the humanized and affinity matured antibodies were demonstrated to reduce or inhibit VEGF activity in different types of *in vitro* assays, and thus act as VEGF antagonists.

The antibodies included within the scope of the invention thus include variant antibodies, such as chimeric (including "humanized") antibodies and hybrid antibodies comprising immunoglobulin chains capable of binding hVEGF, hVEGF_r, or hVEGF-hVEGF_r complex, and a non- hVEGF epitope.

The antibodies herein include all species of origin, and immunoglobulin classes (e.g., IgA, IgD, IgE, IgG, and IgM) and subclasses, as well as antibody fragments (e.g., Fab, F(ab')₂, and Fv), so long as they are capable of binding hVEGF, hVEGF_r, or hVEGF-hVEGF_r complex, and are capable of antagonizing a biological activity of hVEGF.

In a preferred embodiment of the invention, the antibody will have an affinity for the immunizing antigen of at least about 10⁹ liters/mole, as determined, for example, by the Scatchard analysis of Munson & Pollard, Anal. Biochem. 107:220 (1980). Also, the monoclonal antibody typically will inhibit the mitogenic or angiogenic activity of hVEGF at least about 50%, preferably greater than 80%, and most preferably greater than 90%, as determined, for example, by an *in vitro* cell survival or

proliferation assay, such as described in Example 2 or as described in WO 98/45331 and WO 98/45332.

For some therapeutic and diagnostic applications, it is desirable that the monoclonal antibody be reactive with fewer than all of the different molecular forms of hVEGF. For example, it may be desirable to have a monoclonal antibody that is capable of specifically binding to the 165-amino acid sequence hVEGF but not to the 121- or 189-amino acid sequence hVEGF polypeptides. Such antibodies are readily identified by comparative ELISA assays or comparative immunoprecipitation of the different hVEGF polypeptides.

Conjugates with Cytotoxic Moieties

In some embodiments it is desirable to provide a cytotoxic moiety conjugated to a hVEGF-specific monoclonal antibody or to hVEGFr. In these embodiments the cytotoxin serves to incapacitate or kill cells which are expressing or binding hVEGF or its receptor. The conjugate is targeted to the cell by the domain which is capable of binding to hVEGF, hVEGFr, or hVEGF-hVEGFr complex. Thus, monoclonal antibodies that are capable of binding hVEGF, hVEGFr, or hVEGF-hVEGFr complex are conjugated to cytotoxins. Similarly, hVEGFr is conjugated to a cytotoxin. While the monoclonal antibodies optimally are capable of neutralizing the activity of hVEGF alone (without the cytotoxin), it is not necessary in this embodiment that the monoclonal antibody or receptor be capable of any more than binding to hVEGF, hVEGFr, or hVEGF-hVEGFr complex.

Typically, the cytotoxin is a protein cytotoxin, e.g. diphtheria, ricin or Pseudomonas toxin, although in the case of certain classes of immunoglobulins the Fc domain of the monoclonal antibody itself may serve to provide the cytotoxin (e.g., in the case of IgG2 antibodies, which are capable of fixing complement and participating in antibody-dependent cellular cytotoxicity (ADCC)). However, the cytotoxin does not need to be proteinaceous and may include chemotherapeutic agents heretofore employed, for example, for the treatment of tumors.

The cytotoxin typically is linked to a monoclonal antibody or fragment thereof by a backbone amide bond within (or in place of part or all of) the Fc domain of the antibody. Where the targeting function is supplied by hVEGFr, the cytotoxic moiety is substituted onto any domain of the receptor that does not participate in hVEGF binding; preferably, the moiety is substituted in place of or onto the transmembrane and or

cytoplasmic domains of the receptor. The optimal site of substitution will be determined by routine experimentation and is well within the ordinary skill.

Conjugates which are protein fusions are easily made in recombinant cell culture by expressing a gene encoding the conjugate. Alternatively, the conjugates are made by covalently crosslinking the cytotoxic moiety to an amino acid residue side chain or C-terminal carboxyl of the antibody or the receptor, using methods known per se such as disulfide exchange or linkage through a thioester bond using for example iminothiolate and methyl-4-mercaptobutyrimadate.

Conjugates with other Moieties

The monoclonal antibodies and hVEGFr that are antagonists of hVEGF also can also be conjugated to substances that may not be readily classified as cytotoxins in their own right, but which augment the activity of the compositions herein. For example, monoclonal antibodies or hVEGFr capable of binding to hVEGF, hVEGFr, or hVEGF-hVEGFr complex are fused with heterologous polypeptides, such as viral sequences, with cellular receptors, with cytokines such as TNF, interferons, or interleukins, with polypeptides having procoagulant activity, and with other biologically or immunologically active polypeptides. Such fusions are readily made by recombinant methods. Typically such non-immunoglobulin polypeptides are substituted for the constant domain(s) of an anti-hVEGF or anti-hVEGF-hVEGFr complex antibody, or for the transmembrane and/or intracellular domain of an hVEGFr. Alternatively, they are substituted for a variable domain of one antigen binding site of an anti-hVEGF antibody described herein.

In preferred embodiments, such non-immunoglobulin polypeptides are joined to or substituted for the constant domains of an antibody described herein. Bennett, et al., J. Biol. Chem. 266:23060-23067 (1991). Alternatively, they are substituted for the Fv of an antibody herein to create a chimeric polyvalent antibody comprising at least one remaining antigen binding site having specificity for hVEGF, hVEGFr, or a hVEGF-hVEGFr complex, and a surrogate antigen binding site having a function or specificity distinct from that of the starting antibody.

Heterospecific Antibodies

Monoclonal antibodies capable of binding to hVEGF, hVEGFr, or hVEGF-hVEGFr complex need only contain a single binding site for the enumerated epitopes, typically a single heavy-light chain complex or
5 fragment thereof. However, such antibodies optionally also bear antigen binding domains that are capable of binding an epitope not found within any one of hVEGF, hVEGFr, or hVEGF-hVEGFr complex. For example, substituting the corresponding amino acid sequence or amino acid residues of a native anti- hVEGF, anti- hVEGFr, or anti-hVEGF-hVEGFr complex
10 antibody with the complementarity-determining and, if necessary, framework residues of an antibody having specificity for an antigen other than hVEGF, hVEGFr, or hVEGF-hVEGFr complex will create a polyspecific antibody comprising one antigen binding site having specificity for hVEGF, hVEGFr, or hVEGF-hVEGFr complex, and another antigen binding site
15 having specificity for the non- hVEGF, hVEGFr, or hVEGF-hVEGFr complex antigen. These antibodies are at least bivalent, but may be polyvalent, depending upon the number of antigen binding sites possessed by the antibody class chosen. For example, antibodies of the IgM class will be polyvalent.

20 In preferred embodiments of the invention such antibodies are capable of binding an hVEGF or hVEGFr epitope and either (a) a polypeptide active in blood coagulation, such as protein C or tissue factor, (b) a cytotoxic protein such as tumor necrosis factor (TNF), or (c) a non- hVEGFr cell surface receptor, such as CD4, or HER-2 receptor
25 (Maddon, et al., Cell 42:93 (1985); Coussens, et al., Science 230:1137 (1985)). Heterospecific, multivalent antibodies are conveniently made by cotransforming a host cell with DNA encoding the heavy and light chains of both antibodies and thereafter recovering, by immunoaffinity chromatography or the like, the proportion of expressed antibodies having
30 the desired antigen binding properties. Alternatively, such antibodies are made by in vitro recombination of monospecific antibodies.

Monovalent Antibodies

Monovalent antibodies capable of binding to hVEGFr or hVEGF-hVEGFr
35 complex are especially useful as antagonists of hVEGF. Without limiting the invention to any particular mechanism of biological activity, it is believed that activation of cellular hVEGF receptors proceeds by a

mechanism wherein the binding of hVEGF to cellular hVEGF receptors induces aggregation of the receptors, and in turn activates intracellular receptor kinase activity. Because monovalent anti- hVEGF receptor antibodies cannot induce such aggregation, and therefore cannot activate hVEGF receptor by that mechanism, they are ideal antagonists of hVEGF.

It should be noted, however, that these antibodies should be directed against the hVEGF binding site of the receptor or should otherwise be capable of interfering with hVEGF binding to the receptor hVEGF, such as by sterically hindering hVEGF access to the receptor. As described elsewhere herein, however, anti- hVEGFr antibodies that are not capable of interfering with hVEGF binding are useful when conjugated to non-immunoglobulin moieties, for example, cytotoxins.

Methods for preparing monovalent antibodies are well known in the art. For example, one method involves recombinant expression of immunoglobulin light chain and modified heavy chain. The heavy chain is truncated generally at any point in the Fc region so as to prevent heavy chain crosslinking. Alternatively, the relevant cysteine residues are substituted with another amino acid residue or are deleted so as to prevent crosslinking. In vitro methods are also suitable for preparing monovalent antibodies. For example, Fab fragments are prepared by enzymatic cleavage of intact antibody.

Diagnostic Uses

For diagnostic applications, the antibodies or hVEGFr of the invention typically will be labeled with a detectable moiety. The detectable moiety can be any one which is capable of producing, either directly or indirectly, a detectable signal. For example, the detectable moiety may be a radioisotope, such as ^3H , ^{14}C , ^{32}P , ^{35}S , or ^{125}I , a fluorescent or chemiluminescent compound, such as fluorescein isothiocyanate, rhodamine, or luciferin; radioactive isotopic labels, such as, e.g., ^{125}I , ^{32}P , ^{14}C , or ^3H , or an enzyme, such as alkaline phosphatase, beta-galactosidase or horseradish peroxidase.

Any method known in the art for separately conjugating the antibody or hVEGFr to the detectable moiety may be employed, including those methods described by Hunter, et al., Nature 144:945 (1962); David, et al., Biochemistry 13:1014 (1974); Pain, et al., J. Immunol. Meth. 40:219 (1981); and Nygren, J. Histochem. and Cytochem. 30:407 (1982). The

antibodies and receptors of the present invention may be employed in any known assay method, such as competitive binding assays, direct and indirect sandwich assays, and immunoprecipitation assays. Zola, Monoclonal Antibodies: A Manual of Techniques, pp.147-158 (CRC Press, Inc., 1987).

Competitive binding assays rely on the ability of a labeled standard (which may be hVEGF or an immunologically reactive portion thereof) to compete with the test sample analyte (hVEGF) for binding with a limited amount of antibody. The amount of hVEGF in the test sample is inversely proportional to the amount of standard that becomes bound to the antibodies or receptors. To facilitate determining the amount of standard that becomes bound, the antibodies or receptors generally are insolubilized before or after the competition, so that the standard and analyte that are bound to the antibodies or receptors may conveniently be separated from the standard and analyte which remain unbound.

Sandwich assays involve the use of two antibodies or receptors, each capable of binding to a different immunogenic portion, or epitope, of the protein to be detected. In a sandwich assay, the test sample analyte is bound by a first antibody or receptor which is immobilized on a solid support, and thereafter a second antibody binds to the analyte, thus forming an insoluble three part complex. David & Greene, U.S. Pat No. 4,376,110. The second antibody or receptor may itself be labeled with a detectable moiety (direct sandwich assays) or may be measured using an anti-immunoglobulin antibody that is labeled with a detectable moiety (indirect sandwich assay). For example, one type of sandwich assay is an ELISA assay, in which case the detectable moiety is an enzyme.

The antibodies or receptor herein also is useful for in vivo imaging, wherein an antibody or hVEGF_r labeled with a detectable moiety is administered to a patient, preferably into the bloodstream, and the presence and location of the labeled antibody or receptor in the patient is assayed. This imaging technique is useful, for example, in the staging and treatment of neoplasms. The antibody or hVEGF_r is labeled with any moiety that is detectable in a mammal, whether by nuclear magnetic resonance, radiology, or other detection means known in the art.

Antagonist Variants of hVEGF

In addition to the antibodies described herein, other useful antagonists of hVEGF include fragments and amino acid sequence variants of native hVEGF that bind to hVEGF receptor but that do not exhibit the biological activity of native hVEGF. For example, such antagonists include fragments and amino acid sequence variants that comprise a receptor binding domain of hVEGF, but that lack a domain conferring biological activity, or that otherwise are defective in activating cellular hVEGF receptors, such as in the case of a fragment or an amino acid sequence variant that is deficient in its ability to induce aggregation or activation of cellular hVEGF receptors. The term "receptor binding domain" refers to the amino acid sequences in hVEGF that are involved in hVEGF receptor binding. The term "biological activity domain" or "domain conferring biological activity" refers to an amino acid sequence in hVEGF that confers a particular biological activity of the factor, such as mitogenic, angiogenic, or vascular permeability activity.

The observation that hVEGF appears to be capable of forming a complex with two or more hVEGFr molecules on the surface of a cell suggests that hVEGF has at least two discrete sites for binding to hVEGFr and that it binds to such cellular receptors in sequential fashion, first at one site and then at the other before activation occurs, in the fashion of growth hormone, prolactin and the like (see e.g., Cunningham, *et al.*, Science 254:821 (1991); deVos, *et al.*, Science 255:306 (1992); Fuh, *et al.*, Science 256:1677 (1992)). Accordingly, antagonist variants of hVEGF are selected in which one receptor binding site of hVEGF (typically the site involved in the initial binding of hVEGF to hVEGFr) remains unmodified (or if modified is varied to enhance binding), while a second receptor binding site of hVEGF typically is modified by nonconservative amino acid residue substitution(s) or deletion(s) in order to render that binding site inoperative.

Receptor binding domains in hVEGF and hVEGF binding domains in hVEGFr are determined by methods known in the art, including X-ray studies, mutational analyses, and antibody binding studies. The mutational approaches include the techniques of random saturation mutagenesis coupled with selection of escape mutants, and insertional mutagenesis. Another strategy suitable for identifying receptor-binding domains in ligands is known as alanine (Ala)-scanning mutagenesis.

Cunningham, et al., Science 244, 1081-1985 (1989). This method involves the identification of regions that contain charged amino acid side chains. The charged residues in each region identified (i.e. Arg, Asp, His, Lys, and Glu) are replaced (one region per mutant molecule) with Ala and the receptor binding of the obtained ligands is tested, to assess the importance of the particular region in receptor binding. A further powerful method for the localization of receptor binding domains is through the use of neutralizing anti- hVEGF antibodies. Kim, et al., Growth Factors 7:53 (1992). Usually a combination of these and similar methods is used for localizing the domains involved in receptor binding.

The term "amino acid sequence variant" used in reference to hVEGF refers to polypeptides having amino acid sequences that differ to some extent from the amino acid sequences of the native forms of hVEGF. Ordinarily, antagonist amino acid sequence variants will possess at least about 70% homology with at least one receptor binding domain of a native hVEGF, and preferably, they will be at least about 80%, more preferably at least about 90% homologous with a receptor binding domain of a native hVEGF. The amino acid sequence variants possess substitutions, deletions, and/or insertions at certain positions within the amino acid sequence of native hVEGF, such that the variants retain the ability to bind to hVEGF receptor (and thus compete with native hVEGF for binding to hVEGF receptor) but fail to induce one or more of the biological effects of hVEGF, such as endothelial cell proliferation, angiogenesis, or vascular permeability.

"Homology" is defined as the percentage of residues in the amino acid sequence variant that are identical with the residues in the amino acid sequence of a receptor binding domain of a native hVEGF after aligning the sequences and introducing gaps, if necessary, to achieve the maximum percent homology and not considering any conservative substitutions as part of the percentage of amino acid homology. Methods and computer programs for the alignment are well known in the art. One such computer program is "Align 2", authored by Genentech, Inc., which was filed with user documentation in the United States Copyright Office, Washington, DC 20559, on December 10, 1991. Those skilled in the art can determine, using routine skill, appropriate parameters for measuring alignment, including any algorithms needed to achieve maximal alignment over the length of the sequences being compared.

Substitutional variants are those that have at least one amino acid residue in a native sequence removed and a different amino acid inserted in its place at the same position. The substitutions may be single, where only one amino acid in the molecule has been substituted, or they may be multiple, where two or more amino acids have been substituted in the same molecule.

Insertional variants are those with one or more amino acids inserted immediately adjacent to an amino acid at a particular position in a native sequence. Immediately adjacent to an amino acid means connected to either the α -carboxy or α -amino functional group of the amino acid.

Deletional variants are those with one or more amino acid residues in a native sequence removed. Ordinarily, deletional variants will have one or two amino acid residues deleted in a particular region of the molecule.

Fragments and amino acid sequence variants of hVEGF are readily prepared by methods known in the art, such as by site directed mutagenesis of the DNA encoding the native factor. The mutated DNA is inserted into an appropriate expression vector, and host cells are then transfected with the recombinant vector. The recombinant host cells and grown in suitable culture medium, and the desired fragment or amino acid sequence variant expressed in the host cells then is recovered from the recombinant cell culture by chromatographic or other purification methods.

Alternatively, fragments and amino acid variants of hVEGF are prepared in vitro, for example by proteolysis of native hVEGF, or by synthesis using standard solid-phase peptide synthesis procedures as described by Merrifield (J. Am. Chem. Soc. 85:2149 (1963)), although other equivalent chemical syntheses known in the art may be used. Solid-phase synthesis is initiated from the C-terminus of the peptide by coupling a protected α -amino acid to a suitable resin. The amino acids are coupled to the peptide chain using techniques well known in the art for the formation of peptide bonds.

Therapeutic Uses

The terms "treating" "treatment", "therapy" and "therapeutic" as used herein refer to curative therapy, prophylactic therapy and preventative therapy.

For therapeutic applications, the antagonists of the invention are administered to a mammal, preferably a human, in an acceptable dosage form, including those that may be administered to a human intravenously as a bolus or by continuous infusion over a period of time, by
5 intramuscular, intraperitoneal, intra-cerebrospinal, subcutaneous, intra-articular, intrasynovial, intradural, intrathecal, oral, topical, or inhalation routes. The antagonists also are suitably administered by intratumoral, peritumoral, intralesional, or perilesional routes, to exert local as well as systemic therapeutic effects. The intraperitoneal
10 route is expected to be particularly useful, for example, in the treatment of ovarian tumors. Intravenous infusion is expected to be particularly useful for instance, in the treatment of cerebral edema.

Such dosage forms encompass carriers that are inherently nontoxic and nontherapeutic. Examples of such carriers include ion exchangers,
15 alumina, aluminum stearate, lecithin, serum proteins, such as human serum albumin, buffer substances such as phosphates, glycine, sorbic acid, potassium sorbate, partial glyceride mixtures of saturated vegetable fatty acids, water, salts, or electrolytes such as protamine sulfate, disodium hydrogen phosphate, potassium hydrogen phosphate, sodium
20 chloride, zinc salts, colloidal silica, magnesium trisilicate, polyvinyl pyrrolidone, cellulose-based substances, and polyethylene glycol. Carriers for topical or gel-based forms of antagonist include polysaccharides such as sodium carboxymethylcellulose or methylcellulose, polyvinylpyrrolidone, polyacrylates, polyoxyethylene-polyoxypropylene-
25 block polymers, polyethylene glycol, and wood wax alcohols. Conventional depot forms can be suitably used. Such forms include, for example, microcapsules, nano-capsules, liposomes, plasters, inhalation forms, nose sprays, sublingual tablets, and sustained-release preparations. The antagonist will typically be formulated in such vehicles at a
30 concentration of about 0.1 mg/ml to 100 mg/ml.

Suitable examples of sustained release preparations include semipermeable matrices of solid hydrophobic polymers containing the antagonist, which matrices are in the form of shaped articles, e.g. films, or microcapsules. Examples of sustained-release matrices include
35 polyesters, hydrogels (for example, poly(2-hydroxyethyl-methacrylate) as described by Langer *et al.*, J. Biomed. Mater. Res. 15:167 (1981) and Langer, Chem. Tech., 12: 98-105 (1982), or poly(vinylalcohol), polylactides (U.S. Pat. No. 3,773,919), copolymers of L-glutamic acid and

gamma ethyl-L-glutamate (Sidman et al., Biopolymers, 22:547 (1983), non-degradable ethylene-vinyl acetate (Langer et al., supra), degradable lactic acid- glycolic acid copolymers such as the Lupron Depot™ (injectable microspheres composed of lactic acid- glycolic acid copolymer and leuprolide acetate), and poly-D-(-)-3-hydroxybutyric acid. While polymers such as ethylene-vinyl acetate and lactic acid- glycolic acid enable release of molecules for over 100 days, certain hydrogels release proteins for shorter time periods. When encapsulated polypeptide antagonists remain in the body for a long time, they may denature or aggregate as a result of exposure to moisture at 37 °C, resulting in a loss of biological activity and possible changes in immunogenicity. Rational strategies can be devised for stabilization depending on the mechanism involved. For example, if the aggregation mechanism is discovered to be intermolecular S-S bond formation through thio-disulfide interchange, stabilization may be achieved by modifying sulfhydryl residues, lyophilizing from acidic solutions, controlling moisture content, using appropriate additives, and developing specific polymer matrix compositions.

Sustained-release hVEGF antagonist compositions also include liposomally entrapped antagonist antibodies or hVEGF_r. Liposomes containing the antagonists are prepared by methods known in the art, such as described in Epstein, et al., Proc. Natl. Acad. Sci. USA, 82:3688 (1985); Hwang, et al., Proc. Natl. Acad. Sci. USA, 77:4030 (1980); U.S. Patent No. 4,485,045; U.S. Patent No. 4,544,545. Ordinarily the liposomes are the small (about 200-800 Angstroms) unilamellar type in which the lipid content is greater than about 30 mol.% cholesterol, the selected proportion being adjusted for the optimal HRG therapy. Liposomes with enhanced circulation time are disclosed in U.S. Patent No. 5,013,556.

Another use of the present invention comprises incorporating an hVEGF antagonist into formed articles. Such articles can be used, for instance, in modulating endothelial cell growth and angiogenesis. In addition, tumor invasion and metastasis may be modulated with these articles.

An appropriate and effective dosage of antagonist will depend on the type of disease or condition to be treated, as defined herein, the severity and course of the disease or condition, whether the antagonists are administered for preventive or therapeutic purposes, previous

therapy, the patient's clinical history and response to the antagonist, and the discretion of the attending physician. An effective dosage of antagonist will typically be that amount of antagonist administered to achieve the maximal of desired amount of inhibition of VEGF biological activity. The antagonist is suitably administered to the patient at one time or over a series of treatments.

The hVEGF antagonists are useful in the treatment of various neoplastic and non- neoplastic diseases and conditions. Neoplasms and related conditions that are amenable to treatment include breast carcinomas, lung carcinomas, gastric carcinomas, esophageal carcinomas, colorectal carcinomas, liver carcinomas, ovarian carcinomas, thecomas, arrhenoblastomas, cervical carcinomas, endometrial carcinoma, endometrial hyperplasia, endometriosis, fibrosarcomas, choriocarcinoma, head and neck cancer, nasopharyngeal carcinoma, laryngeal carcinomas, hepatoblastoma, Kaposi's sarcoma, melanoma, skin carcinomas, hemangioma, cavernous hemangioma, hemangioblastoma, pancreas carcinomas, retinoblastoma, astrocytoma, glioblastoma, Schwannoma, oligodendroglioma, medulloblastoma, neuroblastomas, rhabdomyosarcoma, osteogenic sarcoma, leiomyosarcomas, urinary tract carcinomas, thyroid carcinomas, Wilm's tumor, renal cell carcinoma, prostate carcinoma, abnormal vascular proliferation associated with phakomatoses, and Meigs' syndrome.

In one embodiment, vascularization of tumors is attacked in combination therapy. One or more hVEGF antagonists are administered to tumor-bearing patients at therapeutically effective doses as determined for example by observing necrosis of the tumor or its metastatic foci, if any. This therapy is continued until such time as no further beneficial effect is observed or clinical examination shows no trace of the tumor or any metastatic foci. Other auxiliary agents such as tumor necrosis factor (TNF), alpha-, beta-, or gamma-interferon, anti-HER2 antibody, heregulin, anti- heregulin antibody, D-factor, interleukin-1 (IL-1), interleukin-2 (IL-2), granulocyte-macrophage colony stimulating factor (GM-CSF), or agents that promote microvascular coagulation in tumors, such as anti-protein C antibody, anti-protein S antibody, or C4b binding protein; (see Esmon, et al., PCT Patent Publication No. WO 91/01753, published 21 February 1991), or heat or radiation.

Since the auxiliary agents will vary in their effectiveness it is desirable to compare their impact on the tumor by matrix screening in conventional fashion. The administration of hVEGF antagonist and, for

instance, TNF, can be repeated until the desired clinical effect is achieved. Alternatively, the hVEGF antagonist(s) can be administered together with TNF and, optionally, auxiliary agent(s). In instances where solid tumors are found in the limbs or in other locations

5 susceptible to isolation from the general circulation, the therapeutic agents described herein are administered to the isolated tumor or organ.

In other embodiments, a FGF or platelet-derived growth factor (PDGF) antagonist, such as an anti-FGF or an anti-PDGF neutralizing antibody, is administered to the patient in conjunction with the hVEGF antagonist.

10 Treatment with hVEGF antagonists optimally may be suspended during periods of wound healing or desirable neovascularization.

Non-neoplastic conditions that are amenable to treatment include rheumatoid arthritis, psoriasis, atherosclerosis, diabetic and other retinopathies, retrolental fibroplasia, neovascular glaucoma, age-related
15 macular degeneration, thyroid hyperplasias (including Grave's disease), corneal and other tissue transplantation, chronic inflammation, lung inflammation, nephrotic syndrome, preeclampsia, ascites, pericardial effusion (such as that associated with pericarditis), and pleural effusion.

20 Age-related macular degeneration (AMD) is a leading cause of severe visual loss in the elderly population. The exudative form of AMD is characterized by choroidal neovascularization and retinal pigment epithelial cell detachment. Because choroidal neovascularization is associated with a dramatic worsening in prognosis, the VEGF antagonists
25 of the present invention are expected to be especially useful in reducing the severity of AMD.

Other conditions that are amendable to treatment include edema. Herein, the term "edema" is used in a general sense and includes conditions in the body or accompanying stroke or head injury
30 characterized by an increase in the extravascular tissue water content, either due to increased free extracellular water alone, or in combination with increased intracellular water. The edema may be present in various tissues in the body. In particular, it is contemplated that the hVEGF antagonists may be employed to treat central nervous system (CNS) edema,
35 including cerebral edema, typically characterized by an increase in brain volume, as well as spinal cord or spinal canal edema or other conditions leading to increased intracranial pressure (such as local spinal cord injury). Increase in brain volume can be, for instance, the result of

increased cerebral blood volume and/or increased tissue water content. The term "edema" used herein includes the pathological conditions referred to in the art as vasogenic edema and cytotoxic edema. Typically, the condition referred to as vasogenic edema has been
5 characterized as being associated with the disruption of the blood-brain barrier (BBB) while cytotoxic edema has been characterized as being associated with an intact BBB. Cerebral edema is described generally in the review article, Hariri, Neurosurgical Intensive Care 5:687 (1994).

Edema in a mammal may result from or accompany a variety of
10 pathological conditions or stimuli, including but not limited to, acute hypertension, meningitis, encephalitis, abscess, neoplastic diseases (such as described above) (particularly solid tumors), trauma (such as head injury), hemorrhage, viral infection, cerebral malaria, stroke, radiation, multiple sclerosis, post cardiac arrest, birth asphyxia,
15 glutamate toxicity, encephalopathy, hypoxia, ischemia and renal dialysis.

In particular, the invention contemplates therapy using the hVEGF antagonists to treat cerebral edema, including cerebral edema accompanying neoplasm(s) in the brain and cerebral edema accompanying stroke. In mammals having a neoplasm(s) in brain tissue, it is common
20 for the mammal to develop or experience cerebral edema. It is contemplated that the hVEGF antagonists of the present invention can be administered, alone or in combination with other therapies, like chemotherapy or radiation therapy administered to treat the brain neoplasm, to reduce or inhibit such edema in the brain.

It is also common for mammals having or having undergone stroke to develop or experience cerebral edema. The term stroke in the present application is used in a general sense and includes the clinical conditions known to the skilled practitioner as ischemic stroke and hemorrhagic stroke. It is recognized within the art that stroke in a
30 patient may be characterized or classified as various particular types of stroke, depending for instance, upon the etiology or pathology of the interruption of blood flow, the types of cells or tissues affected, and the presence of blood extravasation into tissue (such as brain tissue). The different types of stroke that have been clinically characterized
35 include but are not limited to, thrombotic stroke, embolic stroke, hemodynamic stroke, lacunar stroke, and hemorrhagic strokes derived or resulting from intracerebral, subarachnoid, intraventricular, or subdural hemorrhage. The skilled medical practitioner will readily appreciate and

understand the nature of such stroke conditions, and be able to detect and diagnosis the presence or symptoms of such conditions in patients. The present inventive methods contemplate that the hVEGF antagonist molecules can be used in the treatment of all such stroke conditions, particularly to reduce or inhibit edema and protect against cell and tissue damage. The hVEGF antagonists can be administered as an acute treatment following stroke onset to reduce or inhibit for instance, cerebral edema, thereby enhancing the mammal's recovery from the stroke.

The use of the hVEGF antagonists are beneficial in that the treatment may prevent or avoid having to perform surgery (like a craniotomy) on the mammal to reduce or alleviate intracranial pressure due to excess water accumulation in brain tissues. It is also contemplated that upon reduction or prevention of such edema by the hVEGF antagonists, there will be a reduction (i.e., protective effect) in the amount of brain and neuronal tissue that can typically be damaged by intracranial pressure and edema.

Depending on the type and severity of the disease or condition being treated, about 1 μ g/kg to 15 mg/kg of antagonist is an initial candidate dosage for administration to the patient, whether, for example, by one or more separate administrations, or by continuous infusion. A typical daily dosage might range from about 1 μ g/kg to 100 mg/kg or more, depending on the factors mentioned above. For repeated administrations over several days or longer, depending on the condition, the treatment is repeated until a desired suppression of disease symptoms occurs. However, other dosage regimens may be useful. For instance, in the methods of treating cerebral edema or stroke, it may be desirable to administer the hVEGF antagonist(s) immediately upon detection or diagnosis in the patient, within several hours of injury or onset of stroke, or within 1 to 4 days thereafter. The desired administration protocol will typically be within the discretion of the medical practitioner. The progress of the hVEGF antagonist therapy is easily monitored by conventional techniques and assays, including, for example, radiographic techniques (in particular, magnetic resonance imaging, MRI) for neoplastic conditions and edema formation associated with trauma or stroke, or monitoring intracranial pressure for cerebral edema.

According to another embodiment of the invention, the effectiveness of the antagonist in preventing or treating a condition or disease may be improved by administering the antagonist serially or in combination with

another agent that is effective for those purposes, such as tumor necrosis factor (TNF), an antibody capable of inhibiting or neutralizing the angiogenic activity of acidic or basic fibroblast growth factor (FGF) or hepatocyte growth factor (HGF), an antibody capable of inhibiting or neutralizing the coagulant activities of tissue factor, protein C, or protein S (see Esmon, et al., PCT Patent Publication No. WO 91/01753, published 21 February 1991), an antibody capable of binding to HER2 receptor (see Hudziak, et al., PCT Patent Publication No. WO 89/06692, published 27 July 1989), or one or more conventional therapeutic agents such as, for example, alkylating agents, folic acid antagonists, anti-metabolites of nucleic acid metabolism, antibiotics, pyrimidine analogs, 5-fluorouracil, cisplatin, purine nucleosides, amines, amino acids, triazol nucleosides, or corticosteroids. Such other agents may be present in the composition being administered or may be administered separately. Particularly in the treatment of edema or stroke, the antagonist may be administered serially or in combination with agents such as antiviral, antifungal or antiparasitic agents, antibiotics, thrombolytic agents (such as t-PA), osmotic therapy agents (e.g., mannitol), or steroids (like Decadron or prednisone). Use of such agents in combination with the antagonist will be within the ordinary skill of the medical practitioner, and of course, selection of such agents would depend, for instance, on the disease or condition being treated.

In a further method of treatment provided in the present application, it is contemplated that the hVEGF antagonist may be administered serially with hVEGF, particularly in the treatment of stroke. Upon diagnosis or detection of stroke, the hVEGF antagonist may be administered immediately or within approximately 1 to 4 days after onset of the stroke. It is believed that following completion of the administration of the antagonist to reduce or inhibit edema formation, it may be beneficial to administer to the patient an amount of hVEGF sufficient to stimulate or promote re-vascularization. Preferably, the hVEGF would be a recombinant form of hVEGF and would be administered in a pharmaceutically-acceptable carrier.

35

Other Uses

The anti- hVEGF antibodies of the invention also are useful as affinity purification agents. In this process, the antibodies against hVEGF are immobilized on a suitable support, such a Sephadex resin or

filter paper, using methods well known in the art. The immobilized antibody then is contacted with a sample containing the hVEGF to be purified, and thereafter the support is washed with a suitable solvent that will remove substantially all the material in the sample except the hVEGF, which is bound to the immobilized antibody. Finally, the support is washed with another suitable solvent, such as glycine buffer, pH 5.0, that will release the hVEGF from the antibody.

The following examples are offered by way of illustration only and are not intended to limit the invention in any manner. All patent and literature citations in the specification are hereby incorporated by reference in their entirety.

EXAMPLES

Commercially available reagents referred to in the examples were used according to manufacturer's instructions unless otherwise indicated.

The source of those cells identified in the following examples, and throughout the specification, by ATCC accession numbers is the American Type Culture Collection, Manassas, Virginia.

20

EXAMPLE 1

Preparation of Anti- hVEGF Monoclonal Antibodies

To obtain hVEGF conjugated to keyhole limpet hemocyanin (KLH) for immunization, recombinant hVEGF (165 amino acids), Leung, *et al.*, Science 246:1306 (1989), was mixed with KLH at a 4:1 ratio in the presence of 0.05% glutaraldehyde and the mixture was incubated at room temperature for 3 hours with gentle stirring. The mixture then was dialyzed against phosphate buffered saline (PBS) at 4° C. overnight.

Balb/c mice were immunized four times every two weeks by intraperitoneal injections with 5 µg of hVEGF conjugated to 20 µg of KLH, and were boosted with the same dose of hVEGF conjugated to KLH four days prior to cell fusion.

Spleen cells from the immunized mice were fused with P3X63Ag8U.1 myeloma cells, Yelton, *et al.*, Curr. Top. Microbiol. Immunol. 81:1 (1978), using 35% polyethylene glycol (PEG) as described. Yarmush, *et al.*, Proc. Nat. Acad. Sci. 77:2899 (1980). Hybridomas were selected in HAT medium.

Supernatants from hybridoma cell cultures were screened for anti-hVEGF antibody production by an ELISA assay using hVEGF-coated microtiter plates. Antibody that was bound to hVEGF in each of the wells was determined using alkaline phosphatase-conjugated goat anti-mouse IgG immunoglobulin and the chromogenic substrate p- nitrophenyl phosphate. Harlow & Lane, Antibodies: A Laboratory Manual, p.597 (Cold Spring Harbor Laboratory, 1988). Hybridoma cells thus determined to produce anti-hVEGF antibodies were subcloned by limiting dilution, and two of those clones, designated A4.6.1 and B2.6.2, were chosen for further studies.

10

EXAMPLE 2

Characterization of Anti-hVEGF Monoclonal Antibodies

A. Antigen Specificity

The binding specificities of the anti- hVEGF monoclonal antibodies produced by the A4.6.1 and B2.6.2 hybridomas were determined by ELISA. The monoclonal antibodies were added to the wells of microtiter plates that previously had been coated with hVEGF, FGF, HGF, or epidermal growth factor (EGF). Bound antibody was detected with peroxidase conjugated goat anti-mouse IgG immunoglobulins. The results of those assays confirmed that the monoclonal antibodies produced by the A4.6.1 and B2.6.2 hybridomas bind to hVEGF, but not detectably to those other protein growth factors.

B. Epitope Mapping

A competitive binding ELISA was used to determine whether the monoclonal antibodies produced by the A4.6.1 and B2.6.2 hybridomas bind to the same or different epitopes (sites) within hVEGF. Kim, et al., Infect. Immun. 57:944 (1989). Individual unlabeled anti-hVEGF monoclonal antibodies (A4.6.1 or B2.6.2) or irrelevant anti-HGF antibody (IgG1 isotype) were added to the wells of microtiter plates that previously had been coated with hVEGF. Biotinylated anti- hVEGF monoclonal antibodies (BIO-A4.6.1 or BIO-B2.6.2) were then added. The ratio of biotinylated antibody to unlabeled antibody was 1:1000. Binding of the biotinylated antibodies was visualized by the addition of avidin-conjugated peroxidase, followed by o- phenylenediamine dihydrochloride and hydrogen peroxide. The color reaction, indicating the amount of biotinylated antibody bound, was determined by measuring the optical density (O.D) at 495 nm wavelength.

As shown in Figure 1, in each case, the binding of the biotinylated anti-hVEGF antibody was inhibited by the corresponding unlabeled antibody, but not by the other unlabeled anti-hVEGF antibody or the anti-HGF antibody. These results indicate that the monoclonal antibodies produced by the A4.6.1 and B2.6.2 hybridomas bind to different epitopes within hVEGF.

C. Isotyping

The isotypes of the anti- hVEGF monoclonal antibodies produced by the A4.6.1 and B2.6.2 hybridomas were determined by ELISA. Samples of culture medium (supernatant) in which each of the hybridomas was growing were added to the wells of microtiter plates that had previously been coated with hVEGF. The captured anti- hVEGF monoclonal antibodies were incubated with different isotype-specific alkaline phosphatase-conjugated goat anti-mouse immunoglobulins, and the binding of the conjugated antibodies to the anti- hVEGF monoclonal antibodies was determined by the addition of p-nitrophenyl phosphate. The color reaction was measured at 405 nm with an ELISA plate reader.

By that method, the isotype of the monoclonal antibodies produced by both the A4.6.1 and B2.6.2 hybridomas was determined to be IgG1.

D. Binding Affinity

The affinities of the anti- hVEGF monoclonal antibodies produced by the A4.6.1 and B2.6.2 hybridomas for hVEGF were determined by a competitive binding assays. A predetermined sub-optimal concentration of monoclonal antibody was added to samples containing 20,000 - 40,000 cpm ¹²⁵I-hVEGF (1 - 2 ng) and various known amounts of unlabeled hVEGF (1 - 1000 ng). After 1 hour at room temperature, 100 µl of goat anti-mouse Ig antisera (Pel-Freez, Rogers, AR USA) were added, and the mixtures were incubated another hour at room temperature. Complexes of antibody and bound protein (immune complexes) were precipitated by the addition of 500 µl of 6% polyethylene glycol (PEG, mol. wt. 8000) at 4 ° C., followed by centrifugation at 2000 x G. for 20 min. at 4 ° C. The amount of ¹²⁵I-hVEGF bound to the anti-hVEGF monoclonal antibody in each sample was determined by counting the pelleted material in a gamma counter.

Affinity constants were calculated from the data by Scatchard analysis. The affinity of the anti-hVEGF monoclonal antibody produced by the A4.6.1 hybridoma was calculated to be 1.2×10^9 liters/mole. The

affinity of the anti- hVEGF monoclonal antibody produced by the B2.6.2 hybridoma was calculated to be 2.5×10^9 liters/mole.

E. Inhibition of hVEGF Mitogenic Activity

Bovine adrenal cortex capillary endothelial (ACE) cells, Ferrara, et al., Proc. Nat. Acad. Sci. 84:5773 (1987), were seeded at a density of 10^4 cells/ml in 12 multiwell plates, and 2.5 ng/ml hVEGF was added to each well in the presence or absence of various concentrations of the anti-hVEGF monoclonal antibodies produced by the A4.6.1 or B2.6.2 hybridomas, or an irrelevant anti-HGF monoclonal antibody. After culturing 5 days, the cells in each well were counted in a Coulter counter. As a control, ACE cells were cultured in the absence of added hVEGF.

As shown in Figure 2, both of the anti- hVEGF monoclonal antibodies inhibited the ability of the added hVEGF to support the growth or survival of the bovine ACE cells. The monoclonal antibody produced by the A4.6.1 hybridoma completely inhibited the mitogenic activity of hVEGF (greater than about 90% inhibition), whereas the monoclonal antibody produced by the B2.6.2 hybridoma only partially inhibited the mitogenic activity of hVEGF.

F. Inhibition of hVEGF Binding

Bovine ACE cells were seeded at a density of 2.5×10^4 cells/0.5 ml/well in 24 well microtiter plates in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% calf serum, 2 mM glutamine, and 1 ng/ml basic fibroblast growth factor. After culturing overnight, the cells were washed once in binding buffer (equal volumes of DMEM and F12 medium plus 25 mM HEPES and 1% bovine serum albumin) at 4° C.

12,000 cpm 125 I-hVEGF (approx. 5×10^4 cpm/ng/ml) was preincubated for 30 minutes with 5 µg of the anti- hVEGF monoclonal antibody produced by the A4.6.1, B2.6.2, or A2.6.1 hybridoma (250 µl total volume), and thereafter the mixtures were added to the bovine ACE cells in the microtiter plates. After incubating the cells for 3 hours at 4 ° C., the cells were washed 3 times with binding buffer at 4 ° C., solubilized by the addition of 0.5 ml 0.2 N. NaOH, and counted in a gamma counter.

As shown in Figure 3 (upper), the anti- hVEGF monoclonal antibodies produced by the A4.6.1 and B2.6.2 hybridomas inhibited the binding of hVEGF to the bovine ACE cells. In contrast, the anti- hVEGF monoclonal

antibody produced by the A2.6.1 hybridoma had no apparent effect on the binding of hVEGF to the bovine ACE cells. Consistent with the results obtained in the cell proliferation assay described above, the monoclonal antibody produced by the A4.6.1 hybridoma inhibited the binding of hVEGF to a greater extent than the monoclonal antibody produced by the B2.6.2 hybridoma.

As shown in Figure 3 (lower), the monoclonal antibody produced by the A4.6.1 hybridoma completely inhibited the binding of hVEGF to the bovine ACE cells at a 1:250 molar ratio of hVEGF to antibody.

G. Cross-reactivity with other VEGF isoforms

To determine whether the anti-hVEGF monoclonal antibody produced by the A4.6.1 hybridoma is reactive with the 121- and 189-amino acid forms of hVEGF, the antibody was assayed for its ability to immunoprecipitate those polypeptides.

Human 293 cells were transfected with vectors comprising the nucleotide coding sequence of the 121- and 189-amino acid hVEGF polypeptides, as described. Leung, *et al.*, Science 246:1306 (1989). Two days after transfection, the cells were transferred to medium lacking cysteine and methionine. The cells were incubated 30 minutes in that medium, then 100 μ Ci/ml of each 35 S-methionine and 35 S-cysteine were added to the medium, and the cells were incubated another two hours. The labeling was chased by transferring the cells to serum free medium and incubating three hours. The cell culture media were collected, and the cells were lysed by incubating for 30 minutes in lysis buffer (150 mM NaCl, 1% NP40, 0.5% deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 50 mM Tris, pH 8.0). Cell debris was removed from the lysates by centrifugation at 200 x G. for 30 minutes.

500 μ l samples of cell culture media and cell lysates were incubated with 2 μ l of A4.6.1 hybridoma antibody (2.4 mg/ml) for 1 hour at 4° C., and then were incubated with 5 μ l of rabbit anti-mouse IgG immunoglobulin for 1 hour at 4° C. Immune complexes of 35 S-labeled hVEGF and anti-hVEGF monoclonal antibody were precipitated with protein-A Sepharose (Pharmacia), then subjected to SDS - 12% polyacrylamide gel electrophoresis under reducing conditions. The gel was exposed to x-ray film for analysis of the immunoprecipitated, radiolabeled proteins by autoradiography.

The results of that analysis indicated that the anti-hVEGF monoclonal antibody produced by the A4.6.1 hybridoma was cross-reactive with both the 121- and 189-amino acid forms of hVEGF.

5

EXAMPLE 3Preparation of VEGF Receptor - IgG Fusion Proteins**A.**

The nucleotide and amino acid coding sequences of the flt hVEGF receptor are disclosed in Shibuya, et al., Oncogene 5:519-524 (1990).

10 The coding sequence of the entire extracellular domain of the flt hVEGF receptor was fused to the coding sequence of human IgG1 heavy chain in a two-step process.

Site-directed mutagenesis was used to introduce a BstBI restriction into DNA encoding flt at a site 5' to the codon for amino acid 759 of
15 flt, and to convert the unique BstEII restriction site in plasmid pBSSK⁺ FC, Bennett, et al., J. Biol. Chem. 266:23060-23067 (1991), to a BstBI site. The modified plasmid was digested with EcoRI and BstBI and the resulting large fragment of plasmid DNA was ligated together with an
20 EcoRI-BstBI fragment of the flt DNA encoding the extracellular domain (amino acids 1-758) of the flt hVEGF receptor.

The resulting construct was digested with ClaI and NotI to generate an approximately 3.3 kb fragment, which is then inserted into the multiple cloning site of the mammalian expression vector pHEBO2 (Leung, et al., Neuron 8:1045 (1992)) by ligation. The ends of 3.3. kb fragment
25 are modified, for example, by the addition of linkers, to obtain insertion of the fragment into the vector in the correct orientation for expression.

Mammalian host cells (for example, CEN4 cells (Leung, et al. supra) are transfected with the pHEBO2 plasmid containing the flt insert by
30 electroporation. Transfected cells are cultured in medium containing about 10% fetal bovine serum, 2 mM glutamine, and antibiotics, and at about 75% confluency are transferred to serum free medium. Medium is conditioned for 3-4 days prior to collection, and the flt-IgG fusion protein is purified from the conditioned medium by chromatography on a
35 protein-A affinity matrix essentially as described in Bennett, et al., J. Biol. Chem. 266:23060-23067 (1991).

B.

A human flt-IgG (referred to as hflt(1-3)-IgG) cDNA was constructed as described in Davis-Smyth et al., EMBO J. 15:4919-4927 (1996). This truncated receptor form included only the first three immunoglobulin-like domains of human flt fused to a Fc-IgG. See Ferrara et al., Nature Medicine 4:336 (1998).

A murine flt-IgG (referred to as mflt(1-3)-IgG) was constructed by PCR amplification of mouse 17-day embryo cDNA (Clontech, Palo Alto, CA) using primers described in Ferrara et al., supra. The design of the 3' PCR primer ensured that the expression of the mflt-1(1-3) was in frame with a murine IgG2b Fc clone. The resulting 1-kb fragment was first cloned into a TA cloning vector (Invitrogen, San Diego, CA) as a ClaI-BstEII fragment. This fragment was ligated to the 5' end of murine IgG2b Fc in a pRK vector. This plasmid enabled the expression of mflt(1-3)-IgG fusion protein when transfected into mammalian cells.

For expression in CHO cells, the cDNAs were subcloned into a dicistronic vector that links the expression of the marker dihydrofolate reductase to the expression of the flt derived fusion protein. See, Lucas et al., Nucleic Acid Res. 24:1774-1779 (1996). Plasmids were introduced into DP12 cells, a derivative of the CHO-K1DUXB11 cell line developed by L. Chasin (Columbia University, New York) via lipofection and selected for growth in glycine-hypoxanthine-thymidine (G-H-T)-free medium. Chisholm et al., DNA Cloning 4:A Practical Approach, Mammalian Systems (eds. Glover & Hames) pp. 1-39 (Oxford Press, 1995). Clones from the first round of selection were subsequently plated at increasing concentrations of methotrexate. Clones were then screened for production by ELISA for the human or murine Fc. Clones that displayed the highest production were adapted to suspension culture, and serum-free cultures were harvested and purified by protein A-Sepharose. Protein concentrations were determined by amino acid analysis. The endotoxin content of the final purified material did not exceed 0.5 eu/mg.

As described in Ferrara et al., supra, both the murine flt(1-3)-IgG fusion protein and the human flt(1-3)-IgG fusion protein were active in inhibiting bioactivity of VEGF in the tested rodent model.

EXAMPLE 4

Inhibition of Tumor Growth with hVEGF Antagonists

Various human tumor cell lines growing in culture were assayed for production of hVEGF by ELISA. Ovary, lung, colon, gastric, breast, and
5 brain tumor cell lines were found to produce hVEGF. Three cell lines that produced hVEGF, NEG 55 (also referred to as G55) (human glioma cell line obtained from Dr. M. Westphal, Department of Neurosurgery, University Hospital Eppendor, Hamburg, Germany, also referred to as G55), A-673 (human rhabdomyosarcoma cell line obtained from the American Type
10 Culture Collection (ATCC), as cell line number CRL 1598), and SK-LMS-1 (leiomyosarcoma cell line obtained from the ATCC as cell line number HTB 88), were used for further studies.

Six to ten week old female Beige/nude mice (Charles River Laboratory, Wilmington, Massachusetts USA) were injected subcutaneously
15 with $1 - 5 \times 10^6$ tumor cells in 100-200 μ l PBS. At various times after tumor growth was established, mice were injected intraperitoneally once or twice per week with various doses of A4.6.1 anti- hVEGF monoclonal antibody, an irrelevant anti-gp120 monoclonal antibody (5B6), or PBS. Tumor size was measured every week, and at the conclusion of the study
20 the tumors were excised and weighed.

The effect of various amounts of A4.6.1 anti- hVEGF monoclonal antibody on the growth of NEG 55 tumors in mice is shown in Figures 4 and 5. Figure 4 shows that mice treated with 25 μ g or 100 μ g of A4.6.1 anti- hVEGF monoclonal antibody beginning one week after inoculation of NEG 55
25 cells had a substantially reduced rate of tumor growth as compared to mice treated with either irrelevant antibody or PBS. Figure 5 shows that five weeks after inoculation of the NEG 55 cells, the size of the tumors in mice treated with A4.6.1 anti- hVEGF antibody was about 50% (in the case of mice treated with 25 μ g dosages of the antibody) to 85% (in the
30 case of mice treated with 100 μ g dosages of the antibody) less than the size of tumors in mice treated with irrelevant antibody or PBS.

The effect of A4.6.1 anti- hVEGF monoclonal antibody treatment on the growth of SK-LMS-1 tumors in mice is shown in Figure 6. Five weeks after innoculation of the SK-LMS-1 cells, the average size of tumors in
35 mice treated with the A4.6.1 anti- hVEGF antibody was about 75% less than the size of tumors in mice treated with irrelevant antibody or PBS.

The effect of A4.6.1 anti- hVEGF monoclonal antibody treatment on the growth of A673 tumors in mice is shown in Figure 7. Four weeks after inoculation of the A673 cells, the average size of tumors in mice treated with A4.6.1 anti- hVEGF antibody was about 60% (in the case of mice treated with 10 µg dosages of the antibody) to greater than 90% (in the case of mice treated with 50-400 µg dosages of the antibody) less than the size of tumors in mice treated with irrelevant antibody or PBS.

EXAMPLE 5

Analysis of the Direct Effect of Anti-hVEGF Antibody on Tumor Cells Growing in Culture

NEG55 human glioblastoma cells or A673 rhabdomyosarcoma cells were seeded at a density of 7×10^3 cells/well in multiwell plates (12 wells/plate) in F12/DMEM medium containing 10% fetal calf serum, 2mM glutamine, and antibiotics. A4.6.1 anti-hVEGF antibody then was added to the cell cultures to a final concentration of 0 - 20 .0 µg antibody/ml. After five days, the cells growing in the wells were dissociated by exposure to trypsin and counted in a Coulter counter.

Figures 8 and 9 show the results of those studies. As is apparent, the A4.6.1 anti-hVEGF antibody did not have any significant effect on the growth of the NEG55 or A673 cells in culture. These results indicate that the A4.6.1 anti- hVEGF antibody is not cytotoxic, and strongly suggest that the observed anti-tumor effects of the antibody are due to its inhibition of VEGF-mediated neovascularization.

EXAMPLE 6

Effect of Anti-hVEGF Antibody on Endothelial Cell Chemotaxis

Chemotaxis of endothelial cells and others cells, including monocytes and lymphocytes, play an important role in the pathogenesis of rheumatoid arthritis. Endothelial cell migration and proliferation accompany the angiogenesis that occurs in the rheumatoid synovium. Vascularized tissue (pannus) invades and destroys the articular cartilage.

To determine whether hVEGF antagonists interfere with this process, we assayed the effect of the A4.6.1 anti- hVEGF antibody on endothelial cell chemotaxis stimulated by synovial fluid from patients having rheumatoid arthritis. As a control, we also assayed the effect of the

A4.6.1 anti-hVEGF antibody on endothelial cell chemotaxis stimulated by synovial fluid from patients having osteoarthritis (the angiogenesis that occurs in rheumatoid arthritis does not occur in osteoarthritis).

Endothelial cell chemotaxis was assayed using modified Boyden chambers according to established procedures. Thompson, et al., Cancer Res. 51:2670 (1991); Phillips, et al., Proc. Exp. Biol. Med. 197:458 (1991). About 10^4 human umbilical vein endothelial cells were allowed to adhere to gelatin-coated filters (0.8 micron pore size) in 48-well multiwell microchambers in culture medium containing 0.1% fetal bovine serum. After about two hours, the chambers were inverted and test samples (rheumatoid arthritis synovial fluid, osteoarthritis synovial fluid, basic FGF (bFGF) (to a final concentration of 1 $\mu\text{g/ml}$), or PBS) and A4.6.1 anti-hVEGF antibody (to a final concentration of 10 $\mu\text{g/ml}$) were added to the wells. After two to four hours, cells that had migrated were stained and counted.

Figure 10 shows the averaged results of those studies. The values shown in the column labeled "Syn. Fluid" and shown at the bottom of the page for the controls are the average number of endothelial cells that migrated in the presence of synovial fluid, bFGF, or PBS alone. The values in the column labeled "Syn. Fluid + mAB VEGF" are the average number of endothelial cells that migrated in the presence of synovial fluid plus added A4.6.1 anti-hVEGF antibody. The values in the column labeled "% Suppression" indicate the percentage reduction in synovial fluid-induced endothelial cell migration resulting from the addition of anti-hVEGF antibody. As indicated, the anti-hVEGF antibody significantly inhibited the ability of rheumatoid arthritis synovial fluid (53.40 average percentage inhibition), but not osteoarthritis synovial fluid (13.64 average percentage inhibition), to induce endothelial cell migration.

EXAMPLE 7

Effect of VEGF Antagonist on Cerebral Edema

An *in vivo* assay was conducted to determine the effects of a flt- IgG antagonist on cerebral edema. Loss of BBB integrity and the formation of cerebral edema often occurs in the pathogenesis of cerebral infarction. It is believed that breakdown of the BBB in ischemic stroke occurs predominantly after the first 24 hours of stroke onset. Further,

it is believed that the beneficial effects of prompt and adequate restoration of blood flow following an acute ischemic event may be undermined by reperfusion injury to the cerebral microvasculature comprising the BBB, contributing to the formation of cerebral edema.

5 Klatzo et al. Eds., Brain Edema, Tokyo, Springer (1984), pp. 1-5. The *in vivo* assay described below was designed to reflect these aspects of the clinical condition.

Focal cortical ischemia was induced in mouse brain by the occlusion of the middle cerebral artery (MCA) using the techniques previously
10 described by Chen et al., Stroke 17:738-743 (1986). The mice (C57BL-6J; 18-25 grams) were anesthetized with 1.5% isoflurane in oxygen. The right MCA was exposed via a craniotomy and ligated with a 11-0 suture. The ipsilateral common carotid artery was also occluded for the ischemic period. The vessels remained occluded for 45 minutes. Prior to surgery,
15 the animals were randomly divided into two groups and either murine flt-IgG (as described in Example 3B above; also described in Ferrara et al., Nature Medicine 4:336 (1998)) or an irrelevant control murine anti-GP120 antibody belonging to the same isotype as the Fc in the flt-IgG [Ferrara et al., *supra*] was administered intraperitoneally at a dose of 10 mg/kg
20 at 12 hours prior to surgery, at the time of reperfusion and again at 1 and 2 days following surgery. The degree of edema formation was assessed by T2 weighted MR imaging 24 hours following the onset of ischemia. The eventual size of the infarction was assessed 8-12 weeks later using high resolution anatomical MRI. A subset of animals (n=12) were taken for
25 verification of infarction size using conventional histology techniques.

As shown in Fig. 11, administration of flt-Ig caused a significant reduction in the volume of cerebral edema as defined by the region of hyperintensity on the T2-weighted MRI scan acquired 1 day following onset of ischemia (27% reduction, p=0.01 Student's t-test, n=15 and 16 in
30 control and treatment groups, respectively). Representative T2-weighted MR images showing the appearance of cortical edema as a region of high signal intensity compared to the contralateral side is shown in Fig. 12.

In this model, progression of ischemic damage leads to loss of cortical tissue and cavitation. The ultimate infarction volume can, therefore, be
35 estimated from high resolution anatomical images by delineating the amount of unaffected cortex and comparing it to the contralateral hemisphere. As shown in Fig. 13, the size of the cortical infarction is significantly reduced by the administration of flt-IgG measured 8-12

weeks later (26% reduction in infarct size, $p=0.009$ Student's t-test, $n=11$ and 14 in control and treatment groups, respectively). There was a good correlation between the infarct volume measured by MRI and that determined using conventional histology ($R^2=.633$). Accordingly, the

5 treated animals exhibited a reduction in development of cerebral edema, which may further provide enhanced neuroprotection. These results indicate that inhibition of the biological activity of VEGF can reduce ischemic-reperfusion related brain edema and injury.

What is claimed is:

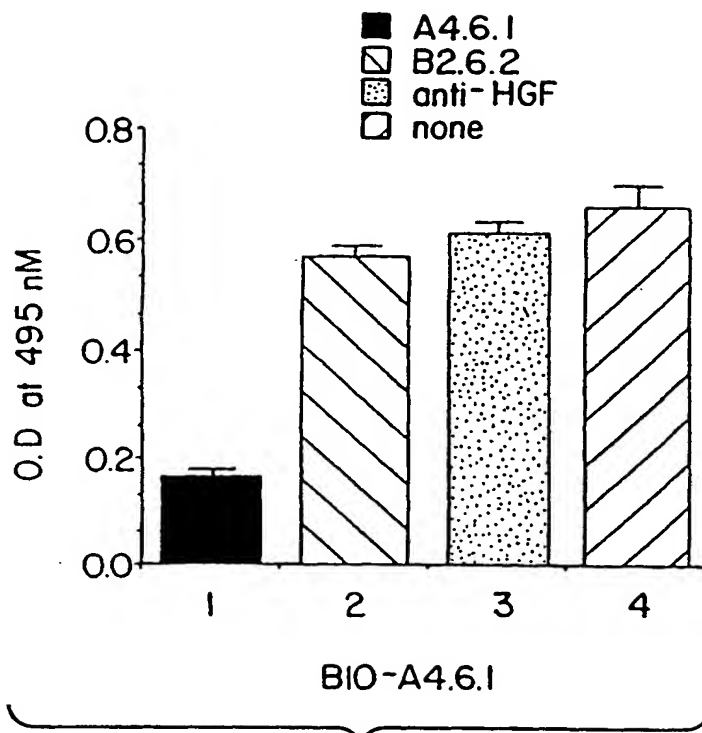
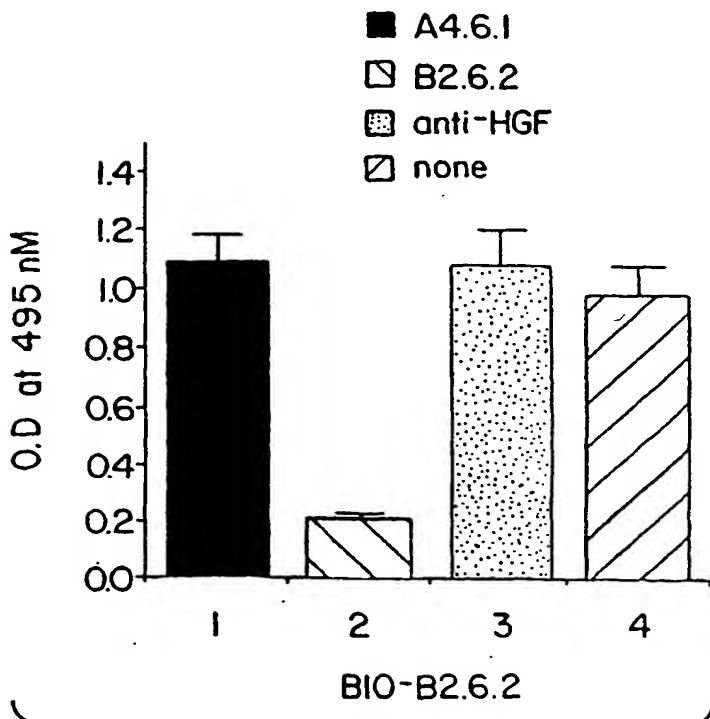
1. A method of treating a mammal having edema comprising administering to said mammal an effective amount of hVEGF antagonist.
- 5 2. The method of claim 1 wherein said edema comprises cerebral edema.
3. The method of claim 1 wherein said mammal is a human further having a neoplastic disease.
- 10 4. The method of claim 3 wherein said neoplastic disease comprises a brain tumor.
5. The method of claim 4 wherein said hVEGF antagonist is administered to said mammal serially or in combination with chemotherapy or radiation
15 therapy.
6. The method of claim 1 wherein said mammal is a human further having or having undergone a stroke.
- 20 7. The method of claim 1 wherein said hVEGF antagonist comprises an anti-hVEGF antibody.
8. The method of claim 7 wherein said anti-hVEGF antibody comprises a chimeric antibody.
25
9. The method of claim 7 wherein said anti-hVEGF antibody comprises a humanized antibody.
10. The method of claim 7 wherein said antibody comprises a monoclonal
30 antibody.
11. The method of claim 1 wherein said hVEGF antagonist comprises a hVEGF receptor fusion protein.
- 35 12. The method of claim 11 wherein said hVEGF receptor fusion protein comprises an extracellular domain sequence of a hVEGF receptor fused to an immunoglobulin.

13. The method of claim 12 wherein said hVEGF receptor fusion protein comprises a flt-IgG fusion protein.
- 5 14. A method of treating a mammal having or having undergone a stroke, comprising administering to said mammal an effective amount of hVEGF antagonist.
- 10 15. The method of claim 14 wherein said hVEGF antagonist comprises an anti-hVEGF antibody.
16. The method of claim 15 wherein said anti-hVEGF antibody comprises a chimeric antibody.
- 15 17. The method of claim 15 wherein said anti-hVEGF antibody comprises a humanized antibody.
18. The method of claim 15 wherein said antibody comprises a monoclonal antibody.
- 20 19. The method of claim 14 wherein said hVEGF antagonist comprises a hVEGF receptor fusion protein.
- 25 20. The method of claim 19 wherein said hVEGF receptor fusion protein comprises an extracellular domain sequence of a hVEGF receptor fused to an immunoglobulin.
- 30 21. The method of claim 20 wherein said hVEGF receptor fusion protein comprises a flt-IgG fusion protein.
22. A method of treating a mammal having cerebral edema comprising administering to said mammal an effective amount of hVEGF antagonist.
- 35 23. The method of claim 22 wherein said hVEGF antagonist comprises an anti-hVEGF antibody.

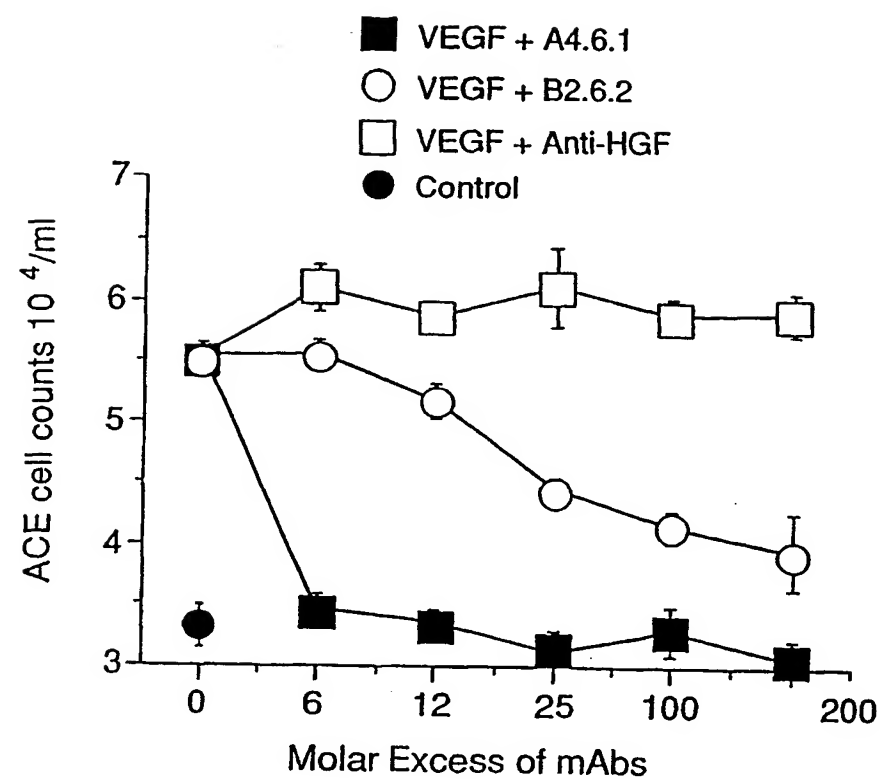
24. The method of claim 23 wherein said anti-hVEGF antibody comprises a chimeric antibody.
25. The method of claim 23 wherein said anti-hVEGF antibody comprises a
5 humanized antibody.
26. The method of claim 23 wherein said antibody comprises a monoclonal antibody.
- 10 27. The method of claim 22 wherein said hVEGF antagonist comprises a hVEGF receptor fusion protein.
28. The method of claim 27 wherein said hVEGF receptor fusion protein
15 comprises an extracellular domain sequence of a hVEGF receptor fused to an immunoglobulin.
29. The method of claim 28 wherein said hVEGF receptor fusion protein
comprises a flt-IgG fusion protein.

20

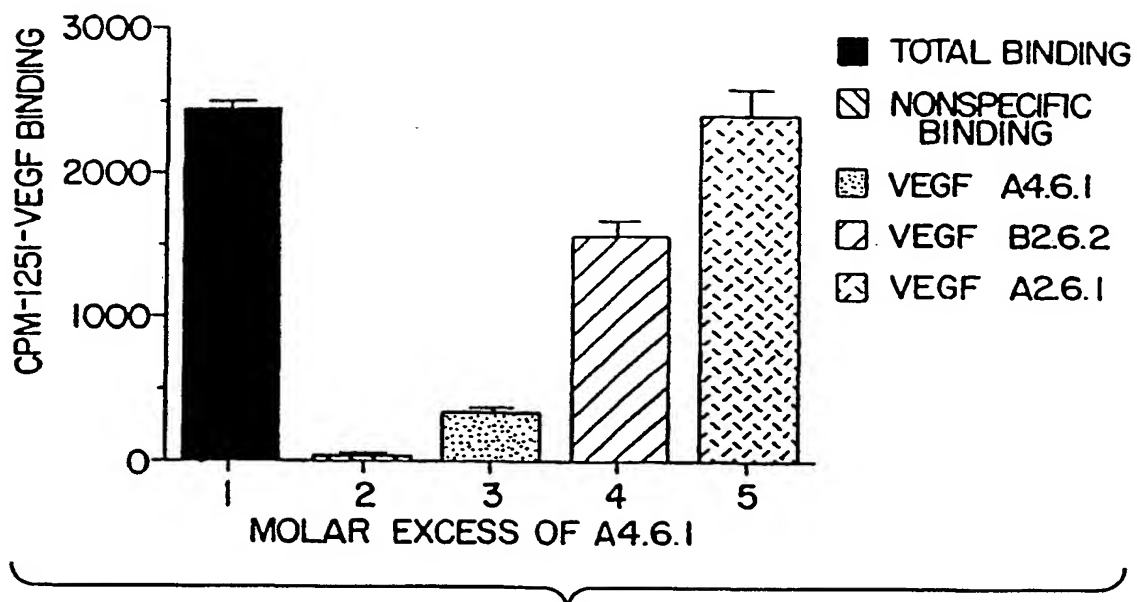
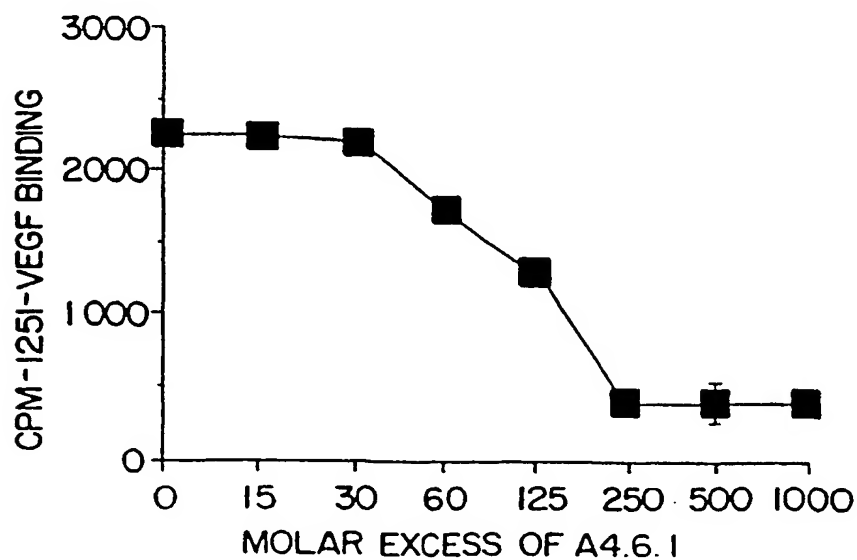
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**FIG. 1a****FIG. 1b**

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**FIG. 2**

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**FIG. 3a****FIG. 3b**

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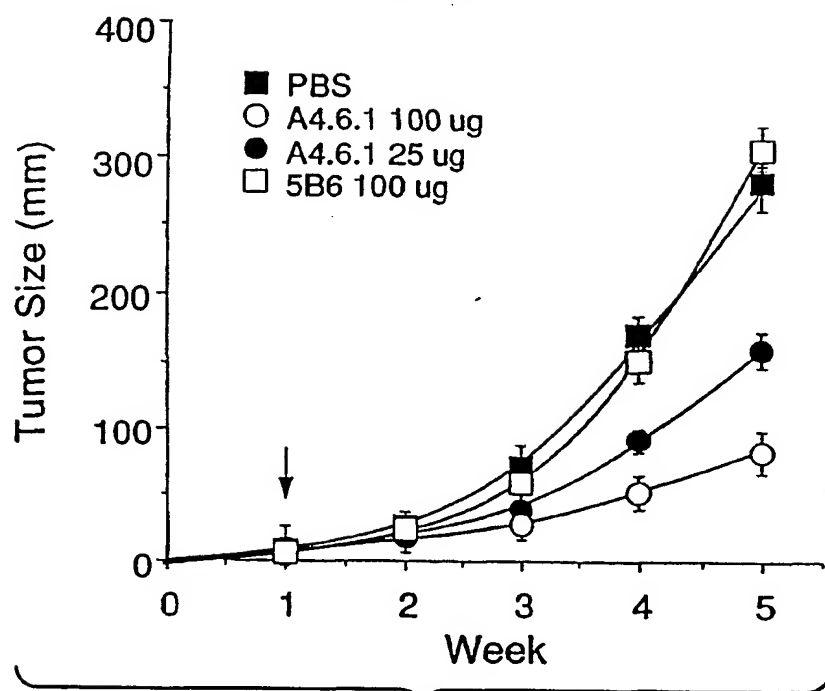


FIG. 4

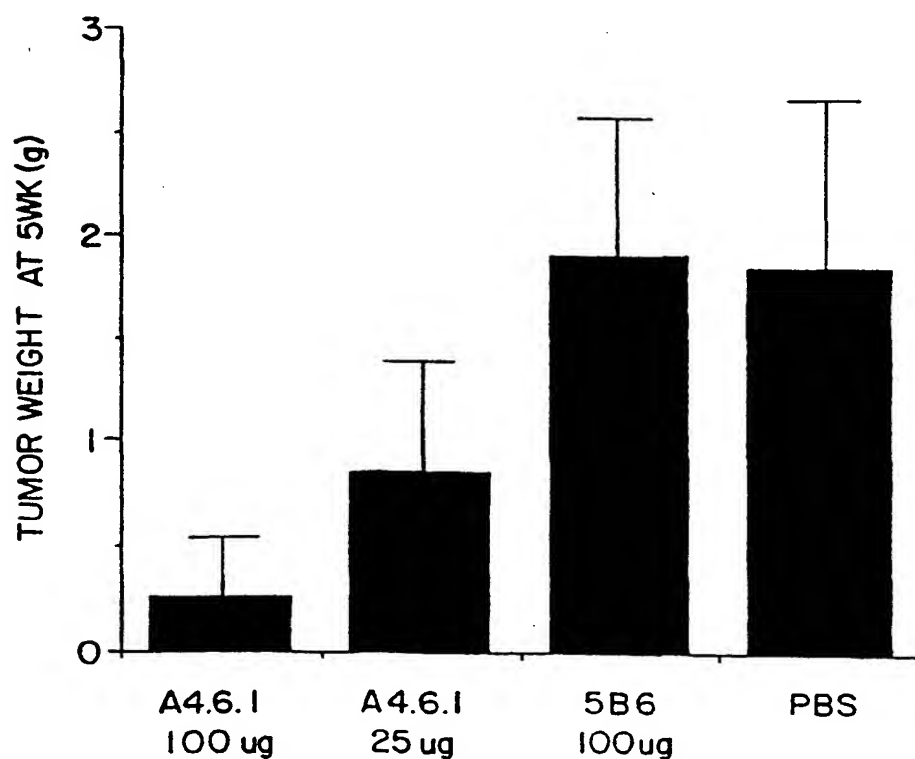


FIG. 5

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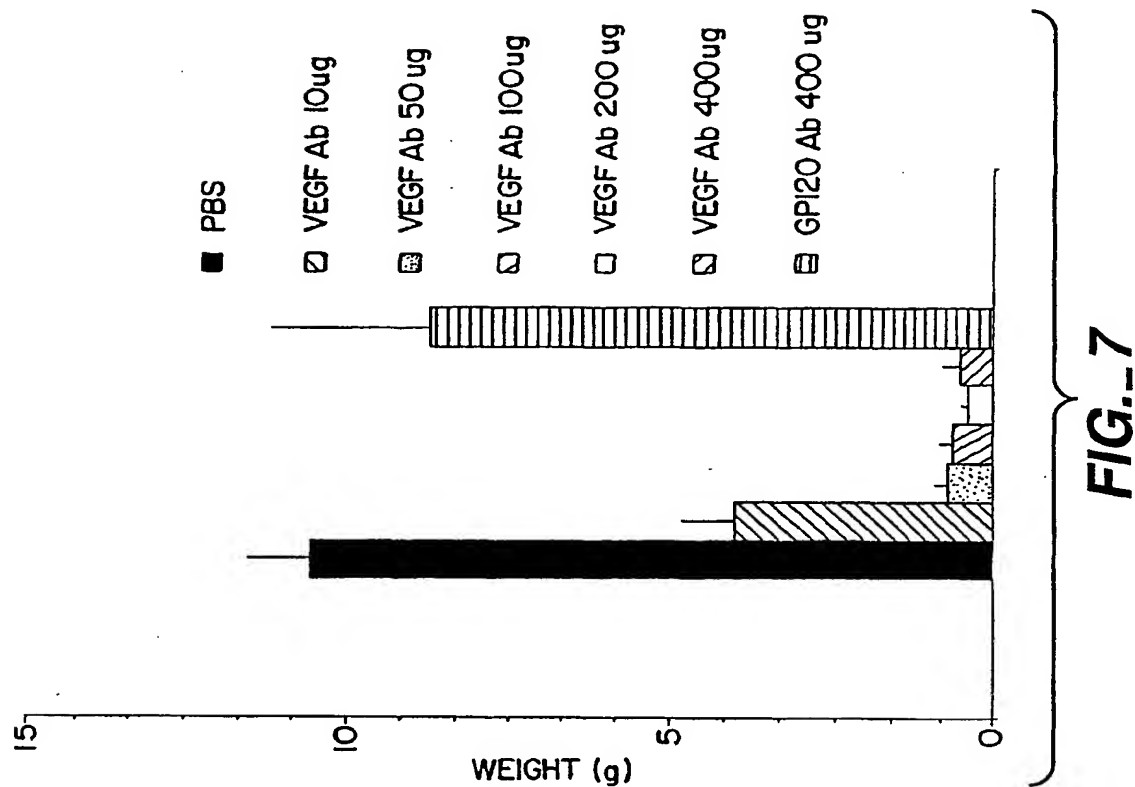


FIG. 7

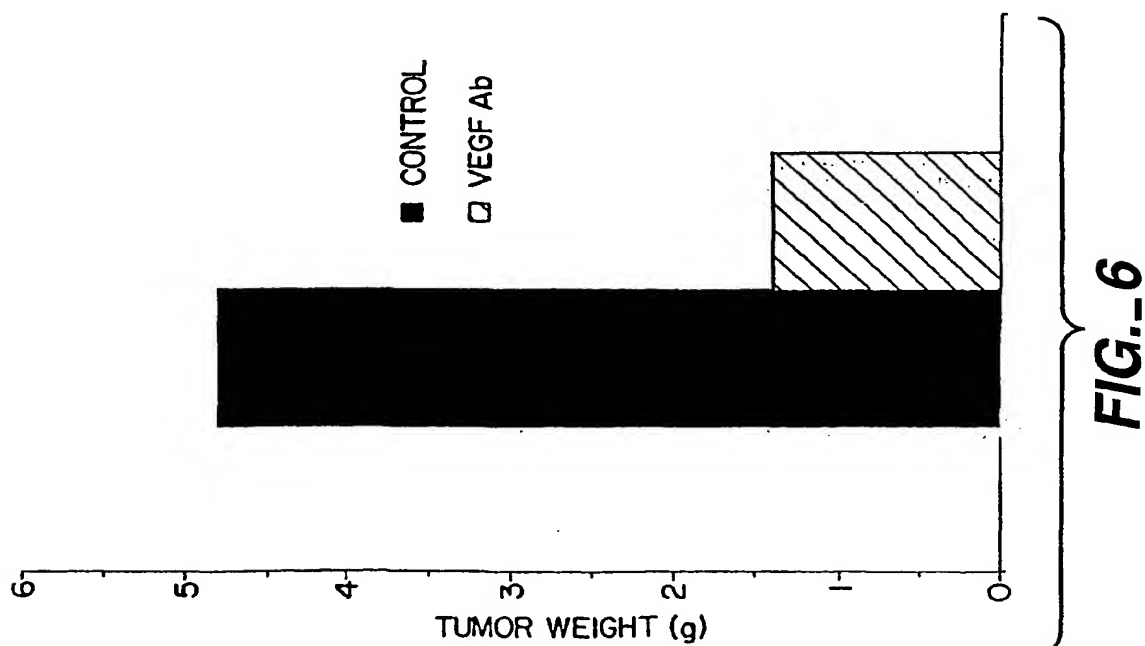
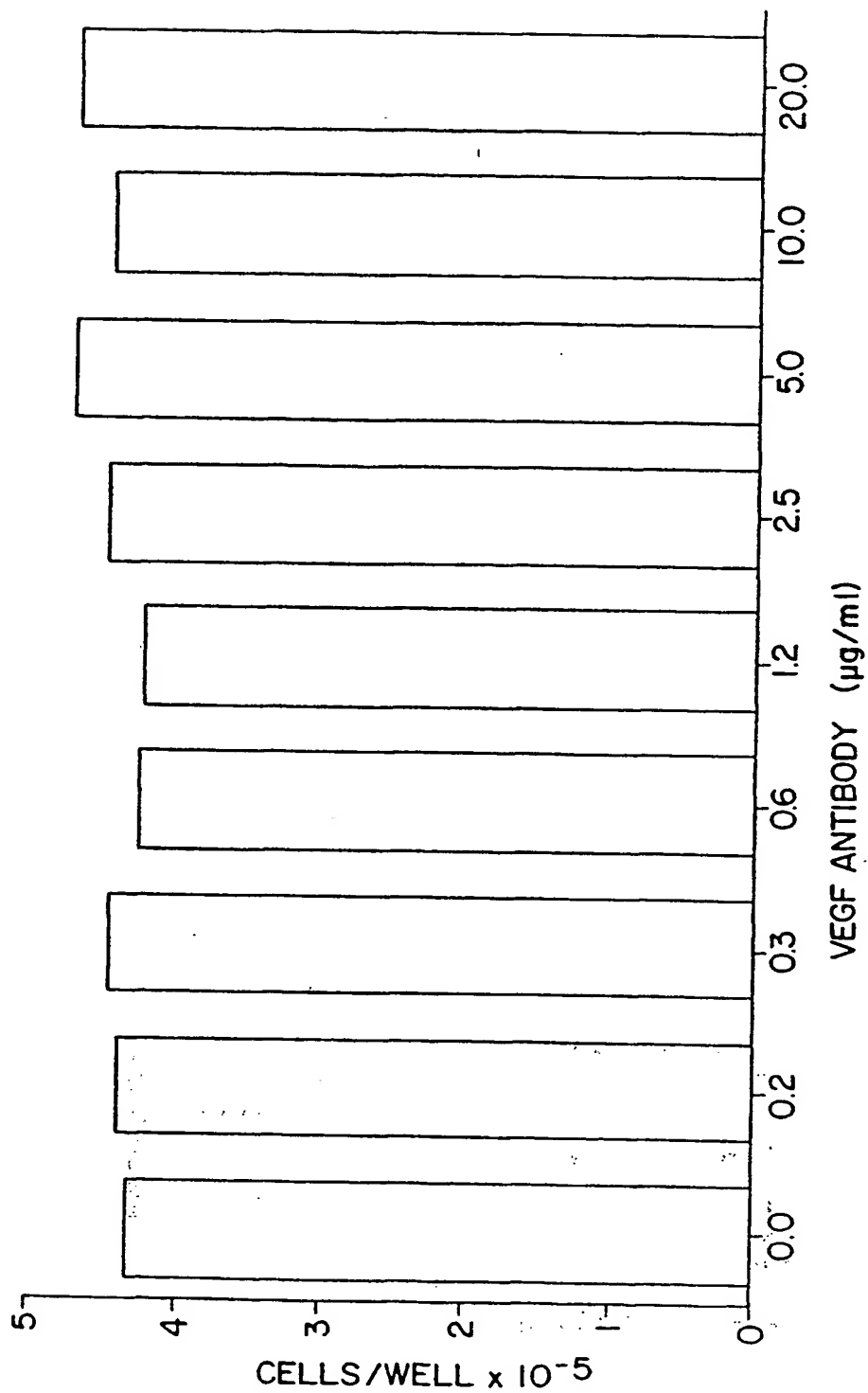


FIG. 6

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**FIG. 8**

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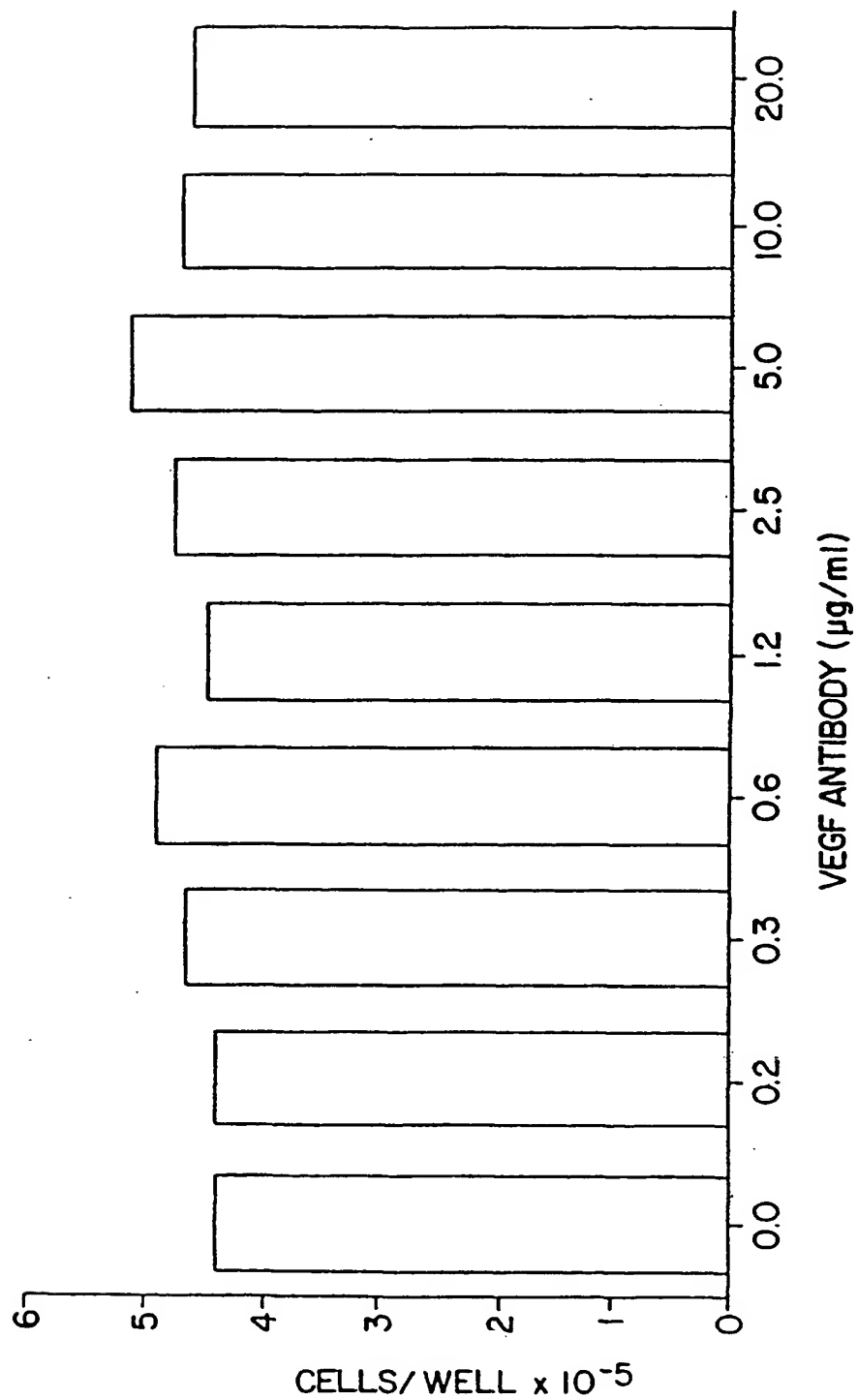


FIG. 9

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Sample Type	Sample #	Assay Date	Syn. Fluid	Syn. Fluid + mAB VEGF	% Suppression
Rheumatoid Syn. Fluid	318	5.7.92	5.2 ± 0.2	2.7 ± 0.3	48
	150	5.7.92	7.0 ± 0.3	2.8 ± 0.4	60
	312	5.7.92	6.7 ± 0.4	3.7 ± 0.3	45
	264	5.7.92	6.2 ± 0.4	3.1 ± 0.3	50
	267	5.7.92	5.7 ± 0.6	4.4 ± 0.3	23
	202	5.22.92	10.0 ± 0.5	3.4 ± 0.6	66
	314	5.22.92	7.5 ± 0.3	3.1 ± 0.6	59
	237	5.22.92	6.1 ± 0.5	2.2 ± 0.3	64
	206	5.22.92	6.7 ± 0.5	2.2 ± 0.3	67
	317	5.22.92	5.2 ± 0.3	2.5 ± 0.6	52
Osteoarthritis Syn. Fluid	165	6.2.92	4.0 ± 0.3	2.8 ± 0.4	30
	211	6.2.92	3.4 ± 0.5	3.0 ± 0.2	11.7
	195	6.2.92	3.5 ± 0.2	3.3 ± 0.3	5.7
	122	6.2.92	3.7 ± 0.3	3.2 ± 0.4	13.5
	16	6.2.92	4.1 ± 0.3	3.8 ± 0.5	7.3

Mean % Suppression for RA Fluids 53.4 ± 4.2
Mean % Suppression for OA Fluids 13.6 ± 3.9
Synovial fluids were diluted 1:50.

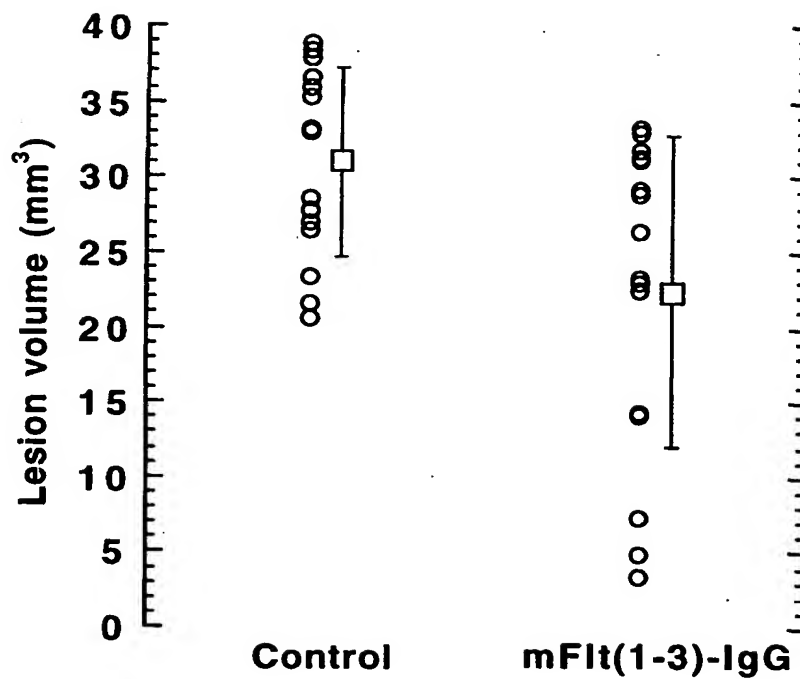
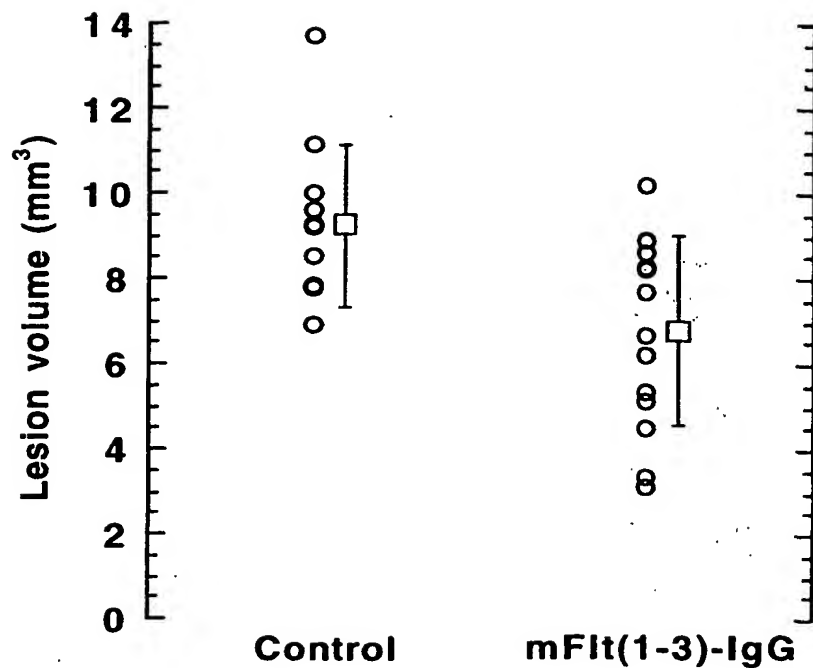
Controls:

6.2.92	PBS	3.3 ± 0.30
	bFGF 1 µg/ml	3.7 ± 0.38
5.22.92	PBS	1.2 ± 0.38
	bFGF 1 µg/ml	7.8 ± 0.31
5.2.92	PBS	1.3 ± 0.18
	bFGF 1 µg/ml	9.0 ± 0.41

FIG. 10

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**FIG. 11****FIG. 13**

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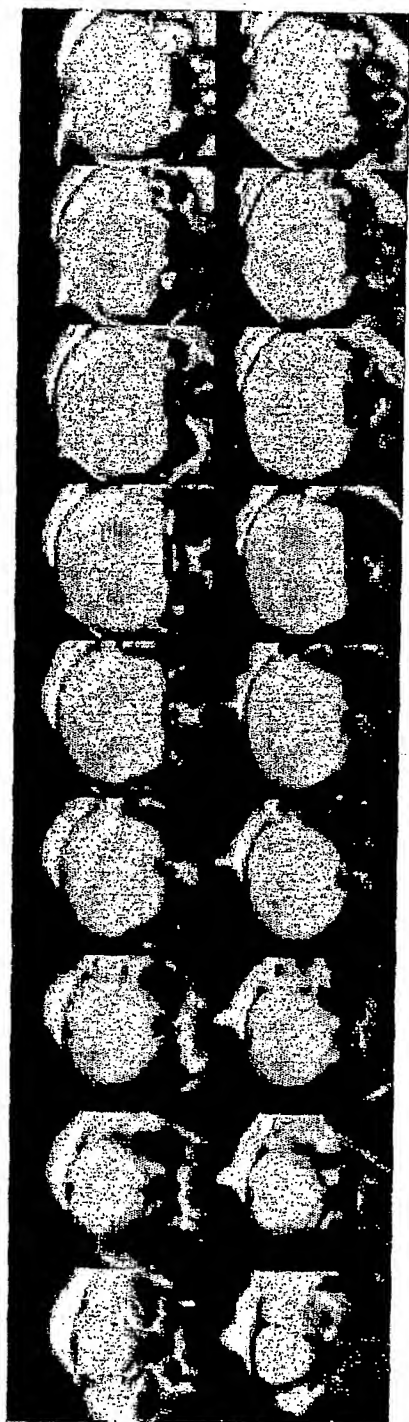


FIG. 12

A

B

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	10	20	30	40
F(ab)-12	DIQMTQSPSS	LSASVGDRV	ITCSASQDIS	NYLNWYQQKP
Y0243-1	DIQMTQSPSS	LSASVGDRV	ITCRANEQLS	NYLNWYQQKP
Y0238-3	DIQMTQSPSS	LSASVGDRV	ITCRANEQLS	NYLNWYQQKP
Y0313-1	DIQMTQSPSS	LSASVGDRV	ITCRANEQLS	NYLNWYQQKP
Y0317	DIQMTQSPSS	LSASVGDRV	ITCSASQDIS	NYLNWYQQKP
CDR-L1				
	50	60	70	80
F(ab)-12	GKAPKVLIIYF	TSSLHSGVPS	RFSGSGSGTD	FTLTISLQ
Y0243-1	GKAPKVLIIYF	TSSLHSGVPS	RFSGSGSGTD	FTLTISLQ
Y0238-3	GKAPKVLIIYF	TSSLHSGVPS	RFSGSGSGTD	FTLTISLQ
Y0313-1	GKAPKVLIIYF	TSSLHSGVPS	RFSGSGSGTD	FTLTISLQ
Y0317	GKAPKVLIIYF	TSSLHSGVPS	RFSGSGSGTD	FTLTISLQ
CDR-L2				
	90	100	110	
F(ab)-12	EDFATYYCQQ	YSTVPWTFGQ	GTKVEIKRTV	
Y0243-1	EDFATYYCQQ	YSTVPWTFGQ	GTKVEIKRTV	
Y0238-3	EDFATYYCQQ	YSTVPWTFGQ	GTKVEIKRTV	
Y0313-1	EDFATYYCQQ	YSTVPWTFGQ	GTKVEIKRTV	
Y0317	EDFATYYCQQ	YSTVPWTFGQ	GTKVEIKRTV	
CDR-L3				

FIG. 14A

	10	20	30	40
F(ab)-12	EVQLVESGGG	LVQPGGSLRL	SCAASGYTFT	NYGMNWRQA
Y0243-1	EVQLVESGGG	LVQPGGSLRL	SCAASGYDFT	HYGMNWRQA
Y0238-3	EVQLVESGGG	LVQPGGSLRL	SCAASGYTFT	NYGMNWRQA
Y0313-1	EVQLVESGGG	LVQPGGSLRL	SCAASGYDFT	HYGMNWRQA
Y0317	EVQLVESGGG	LVQPGGSLRL	SCAASGYDFT	HYGMNWRQA
CDR-H1				
	50	60	70	80
F(ab)-12	PGKGLEWVGW	INTYTGEPTY	AADFKRRFTF	SLDTSKSTAY
Y0243-1	PGKGLEWVGW	INTYTGEPTY	AADFKRRFTF	SLDTSKSTAY
Y0238-3	PGKGLEWVGW	INTYTGEPTY	AADFKRRFTF	SLDTSKSTAY
Y0313-1	PGKGLEWVGW	INTYTGEPTY	AADFKRRFTF	SLDTSKSTAY
Y0317	PGKGLEWVGW	INTYTGEPTY	AADFKRRFTF	SLDTSKSTAY
CDR-H2				
	90	100	110	
F(ab)-12	LQMNSLRAED	TAVYYCAKYP	HYYGSSHWYF	DVWGQGTL
Y0243-1	LQMNSLRAED	TAVYYCAKYP	HYYGSSHWYF	DVWGQGTL
Y0238-3	LQMNSLRAED	TAVYYCAKYP	YYYGTSHWYF	DVWGQGTL
Y0313-1	LQMNSLRAED	TAVYYCAKYP	YYYGTSHWYF	DVWGQGTL
Y0317	LQMNSLRAED	TAVYYCAKYP	YYYGTSHWYF	DVWGQGTL
CDR-H3				
CDR-7				

FIG. 14B

12 / 13

□ = differences from F(ab)-12

F(ab)-12 DIQMTQSPSSLSASVGDRVTITCSASQDISNYLNWYQQ
 Y0192 DI□□TQSPSSLSASVGDRVTITC□RANEOLSNYLNWYQQ
 Y0238-3 DI□□TQSPSSLSASVGDRVTITC□RANEOLSNYLNWYQQ
 Y0239-19 DI□□TQSPSSLSASVGDRVTITC□RANEOLSNYLNWYQQ
 Y0313-2 DI□□TQSPSSLSASVGDRVTITC□RANEOLSNYLNWYQQ

CDR-L1
 F(ab)-12 KPGKAPKVLIIYFTSSLHSGVPSRFSGSGSGTDFTLTIS
 Y0192 KPGKAPKVLIIYFTSSLHSGVPSRFSGSGSGTDFTLTIS
 Y0238-3 KPGKAPKVLIIYFTSSLHSGVPSRFSGSGSGTDFTLTIS
 Y0239-19 KPGKAPKVLIIYFTSSLHSGVPSRFSGSGSGTDFTLTIS
 Y0313-2 KPGKAPKVLIIYFTSSLHSGVPSRFSGSGSGTDFTLTIS

CDR-L2
 F(ab)-12 SLQPEDFATYYCQQYSTVPWTFGQGTKVEIKRTV
 Y0192 SLQPEDFATYYCQQYSTVPWTFGQGTKVEIKRTV
 Y0238-3 SLQPEDFATYYCQQYSTVPWTFGQGTKVEIKRTV
 Y0239-19 SLQPEDFATYYCQQYSTVPWTFGQGTKVEIKRTV
 Y0313-2 SLQPEDFATYYCQQYSTVPWTFGQGTKVEIKRTV

CDR-L3

FIG. 15A

□ = differences from F(ab)-12

	10	20	30	
F(ab)-12	EVQLVESGGGLVQPGGSLRLS	CAASGYTF	TNYGMNWVR	
Y0192	EVQLVESGGGLVQPGGSLRLS	CAASGYTF	TNYGMNWVR	
Y0238-3	EVQLVESGGGLVQPGGSLRLS	CAASGYD	FTNYGMNWVR	
Y0239-19	EVQLVESGGGLVQPGGSLRLS	CAASGYTF	TNYGMNWVR	
Y0313-2	EVQLVESGGGLVQPGGSLRLS	CAASGYD	FTNYGMNWVR	
	40	50	60	70
F(ab)-12	QAPGKGLEWVGWINTYTGEPT	YAADEFKRRFT	FSLDTSKSTA	CDR-H1
Y0192	QAPGKGLEWVGWINTYTGEPT	YAADEFKRRFT	FSLDTSKSTA	
Y0238-3	QAPGKGLEWVGWINTYTGEPT	YAADEFKRRFT	FSLDTSKSTA	
Y0239-19	QAPGKGLEWVGWINTYTGEPT	YAADEFKRRFT	FSLDTSKSTA	
Y0313-2	QAPGKGLEWVGWINTYTGEPT	YAADEFKRRFT	FSLDTSKSTA	
	80	90	100	
F(ab)-12	YLQMNSLRAEDTAVYYCAKYPHYG	---	SSHWFVDVWGQGTLL	CDR-7
Y0192	YLQMNSLRAEDTAVYYCAKYPHYG	---	SSHWFVDVWGQGTLL	
Y0238-3	YLQMNSLRAEDTAVYYCAKYPHYG	---	SSHWFVDVWGQGTLL	
Y0239-19	YLQMNSLRAEDTAVYYCAKYPHYV	NERK	SHWFVDVWGQGTLL	
Y0313-2	YLQMNSLRAEDTAVYYCAKYPHYV	NERK	SHWFVDVWGQGTLL	CDR-H3

FIG. 15B

INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 99/29475

A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 A61K39/395 A61K38/17 A61P7/10 C07K19/00 //C07K14/71

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 C07K A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 94 10202 A (GENENTECH INC) 11 May 1994 (1994-05-11) page 2, line 38 - page 3, line 4 page 5, line 8 - line 16 page 6, line 8 - line 35 page 8, line 24 - line 27 page 16, line 25 - line 37 page 17, line 15 - line 28	1-29
X	WO 98 16551 A (FERRARA NAPOLEONE ; NGUYEN FRANCIS HUNG (US); GENENTECH INC (US); K) 23 April 1998 (1998-04-23) abstract; claims 1-15 page 5, line 15 - line 31 -/-	1-4, 22

☒ Further documents are listed in the continuation of box C.☒ Patent family members are listed in annex.

* Special categories of cited documents :

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"&" document member of the same patent family

Date of the actual completion of the international search

31 May 2000

Date of mailing of the international search report

23.06.00

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von Ballmoos, P

INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 99/29475

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 98 45331 A (CHEN YVONNE MAN YEE ;BACA MANUEL (US); GENENTECH INC (US); WELLS J) 15 October 1998 (1998-10-15) the whole document	1-29
A	WO 97 44453 A (GENENTECH INC ;DAVIS SMYTH TERRI LYNN (US); CHEN HELEN HSIFEI (US)) 27 November 1997 (1997-11-27) page 39; claims 1,10	1-29
A	BERKMAN, RICHARD A. ET AL: "Expression of the vascular permeability factor/vascular endothelial growth factor gene in central nervous system neoplasms." JOURNAL OF CLINICAL INVESTIGATION, (1993) VOL. 91, NO. 1, PP. 153-159. , XP000909359 the whole document	1-29
P,X	VAN BRUGGEN N ET AL: "VEGF antagonism reduces edema formation and tissue damage after ischemia /reperfusion injury in the mouse brain." JOURNAL OF CLINICAL INVESTIGATION, (1999 DEC) 104 (11) 1613-20. , XP000909358 abstract page 1619, right-hand column	1-29

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 99/29475

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
Although claims 1-29 are directed to a method of treatment of the mammal body, the search has been carried out and based on the alleged effects of the compound.
2. ☐ Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

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2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/US 99/29475

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9410202 A	11-05-1994	CA 2145985 A	11-05-1994
		CZ 9501057 A	13-12-1995
		HU 70810 A	28-11-1995
		AU 687727 B	05-03-1998
		AU 2928992 A	24-05-1994
		BG 99605 A	29-02-1996
		BR 9207175 A	12-12-1995
		EP 0666868 A	16-08-1995
		FI 951987 A	26-04-1995
		JP 8502514 T	19-03-1996
		NO 951609 A	27-04-1995
		SK 55195 A	09-08-1995
WO 9816551 A	23-04-1998	AU 718327 B	13-04-2000
		AU 5152398 A	11-05-1998
		EP 0931092 A	28-07-1999
WO 9845331 A	15-10-1998	AU 7100798 A	30-10-1998
		AU 7102398 A	30-10-1998
		EP 0973804 A	26-01-2000
		EP 0971959 A	19-01-2000
		NO 994869 A	06-12-1999
		NO 994870 A	06-12-1999
		WO 9845332 A	15-10-1998
		ZA 9802907 A	09-10-1998
WO 9744453 A	27-11-1997	ZA 9802908 A	21-01-1999
		AU 717112 B	16-03-2000
		AU 3060497 A	09-12-1997
		EP 0907733 A	14-04-1999
		JP 2000502357 T	29-02-2000
		NZ 332779 A	29-06-1999
		US 5952199 A	14-09-1999

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